PB83-264077

Evaluation of the Mutagenicity of Municipal Sewage Sludge

Illinois Univ. at Urbana-Champaign

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

Sep 83

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA (Please read Instructions on the reverse before completing)		
1. REPORT NO. EPA-600/1-83-016	2.	3. RECIPIENT'S ACCESSION NO. PB83 264077
4. TITLE AND SUBTITLE The Evaluation of the Mutagenicity of Municipal Sewage Sludge		5. REPORT DATE September 1983 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Philip K. Hopke & Michael J. Plewa		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Institute for Environmental Studies University of Illinois Urbana, IL 61801		10. PROGRAM ELEMENT NO. CAYBIB 11. CONTRACT/GRANT NO. R807009
12. SPONSORING AGENCY NAME AND ADDRESS U.S. Environmental Protection Agency (TMD) Health Effects Research Laboratory Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES Project Officer: Dr. Norman E. Kowal, Microbiology Branch, TMD, HERL, Cincinnati, OH		

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) unclassified 20. SECURITY CLASS (This page) unclassified	21. NO. OF PAGES 75 22. PRICE

EPA Form 2220-1 (9-73)

PB83-264077

EPA-600/1-83-016
September 1983

THE EVALUATION OF THE MUTAGENICITY OF MUNICIPAL SEWAGE SLUDGE

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory conducts a coordinated environmental health research program in inhalation toxicology, genetic toxicology, neurotoxicology, developmental and experimental biology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, water pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides and other chemical pollutants. The Laboratory participates in and provides data for the development and revision of criteria documents on pollutants for which national ambient air quality and water quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

This report provides information on the multiorganism testing program of five municipal sewage sludges from Illinois cities to determine the presence or absence of mutagenic activity. Mutagenic activity has been observed in some of the sludges, but sludges from primarily domestic sewage do not apparently contain mutagens.

F. G. Hueter, Ph.D.
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ABSTRACT

Samples of five municipal sewage sludges from Illinois cities have been subjected to a multiorganism testing program to determine the presence or absence of mutagenic activity. Chicago sludge has been the most extensively tested using the Salmonella/microsomal activation assay, the micronucleus test in Tradescantia, the wx locus assay in Z. mays, and sister chromatid exchange induction in human lymphocytes. Mutagenic activity has been observed in the sludges from Chicago and Sauget, Illinois. Sludges from primarily domestic sewage do not apparently contain mutagens.

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ACKNOWLEDGEMENTS

We acknowledge the efforts of the people who have contributed to the work reported here: Danny L. Weaver, Dr. Susanne G. Wood, Ray Garton, Sara Engl, Patrick Dowd, and Elizabeth Wagner. We thank our coinvestigators, Professors James B. Johnston and Thomas Hinesly, for their assistance and Professor Richard A. Larson for his advise in developing the separations procedures. We thank Gene Ziegler of the Illinois Agronomy Research Center at Ellwood, Illinois for his care of and attention to the *in situ* experimental plots. Finally, we express our appreciation to the Urbana-Champaign Sanitary District for the sample of Champaign, Illinois sludge and to Luther Landon of the Illinois Environmental Protection Agency for the sludge samples from Hinsdale, Kankakee, and Sauget, Illinois.

SECTION 1

INTRODUCTION

A major problem facing the United States is the disposal of the sludge resulting from the treatment of municipal sewage. There has been a substantial increase in the number and efficiency of sewage treatment plants in the past decade particularly in suburban and rural areas resulting in an increased amount of sludge requiring disposal. In addition, previously employed sludge disposal methods such as ocean dumping are soon to be severely limited or stopped entirely. The incineration of sludge requires expensive facilities and may result in increased air pollution. An attractive alternative disposal method is the application of this organic matter to agricultural lands or its use in the reclamation of land stripped of its organic matter by surface mining.

There may, however, be problems associated with sludge disposal by land application. Analyses of sludge from the Calumet and Southwest treatment plants of the Metropolitan Sanitary District of Chicago showed that concentrations of several heavy metals were substantially enriched over typical soil compositions. Thus, in 1967, a large demonstration and research program was initiated to examine the feasibility of land application of digested sewage sludge from the Metropolitan Sanitary District of Chicago waste water treatment plants. A major objective was to examine compositional changes in soil, crops, and water arising from sludge-amended fields.

In the intervening period, there has been a great deal of study of the uptake and accumulation of heavy metals (most notably zinc and cadmium) in corn, the effects of these elements on soil bacteria, and the potential for transmission of heavy metals through a food chain that might result in impacts on the general public health if widespread use were made of sludge as a soil additive (1-6). It has been found that zinc and cadmium accumulate in corn grain and leaf tissues (5,6). High levels of sludge treatment appear to inhibit the development of several inbred strains of corn but do not cause overt symptoms of metal toxicity (6). Long term studies of food chain and field productivity effects of sludge are in progress.

Additional contaminants that may be present in municipal sewage sludge are organic compounds from industrial processes or other anthropogenic activities. Of potential concern are compounds that are

genetically active and may cause mutations, birth defects, or cancer. In a metropolitan area like Chicago there are major chemical, petrochemical, petroleum-refining, and other industries that utilize organic chemicals in large quantities. Other activities such as fossil fuel combustion produce genotoxic compounds that may find their way into the sanitary sewer system. Therefore, there exists the possibility that there are genotoxic compounds present in the sewage sludge that may be released into the environment by leaching into surface or groundwater or incorporated into the human food chain through the use of sewage sludge as a soil additive. In order to examine this question, samples of sewage sludge were obtained from several local sewage treatment plants that treat a variety of wastes ranging from only household wastes to combinations of industrial and domestic wastes. These sludge samples have been subjected to a multiorganism screening procedure to determine if mutagenic compounds are present in sludge and sludge-amended soil.

SECTION 2

CONCLUSIONS

This study has determined that mutagenic activity is present in some municipal sewage sludges. Sludges from Champaign, Chicago, Hinsdale, Kankakee, and Sauget, Illinois were studied. The Chicago municipal sewage sludge sample clearly has components that induce a variety of mutagenic responses. Although it is not possible to compare the quantitative responses in the various test organisms employed, the sludge or sludge extracts did induce a response in all of the species employed. The supernatant liquid after appropriate concentration was found to be mutagenic using the Ames Salmonella assay. Acetone, hexane, and chloroform:methanol extracts of the pellet contained promutagens that could be activated by mammalian microsomes(S-9). Acetone was a more effective solvent than hexane in the extraction of the promutagens from the sludge pellet. The results indicate that a moderately potent agent can be extracted from the sludge since, with microsomal activation, the material extracted from only 155 µl of whole Chicago sludge was able to induce twice the number of revertant colonies per plate of Ames strain TA98 compared to the background negative control plates.

The Champaign, Hinsdale, and Kankakee showed a weak response from samples obtained by the further chemical fractionation of the chloroform:methanol extracts following activation. Only the Sauget sample demonstrated direct acting mutagenic activity as well as very substantial mutagenicity after activation with mammalian hepatic microsomes.

In this project, the experiments using the Tradescantia micronucleus test encompassed the analysis of over 2.0×10^6 meiotic cells that were individually scored for micronuclei. The project mean negative control frequency based on 2.0×10^5 tetrads was 3.53 MCN/100 tetrads. The overall positive control (50 mm maleic hydrazide) frequency based on 4.7×10^4 tetrads was 10.71 MCN/100 tetrads.

The Tradescantia data clearly indicate that whole or diluted Chicago sludge induces chromosome aberrations in meiotic cells. The aqueous phase is much less mutagenic than whole sludge and it appears that a major portion of the mutagenic agents remain in the sludge solids. The

acetone fraction of the Chicago sludge gave a negative response in the micronucleus test. When whole sludge samples from each municipality except Sauget were evaluated an interesting pattern of response emerged. A ranking in order of decreasing mutagenic response is that of Chicago, Kankakee, Hinsdale and Champaign. When concentrated, whole sludge samples are evaluated the same ranked order is maintained. However, the Chicago sludge is a much more potent mutagen than the Kankakee sludge samples. A 1/2 dilution of whole Chicago sludge induced a mean value of 5.19 MCN/100 tetrads as compared to a value of 6.30 MCN/100 tetrads for the 13.3x concentration of Kankakee whole sludge. Neither the Hinsdale or Champaign sludge samples induced a significant frequency of micronuclei in Tradescantia tetrads. The Sauget sample has not yet been tested with this assay. There is an apparent correlation of the cytogenetic potency of the sludge sample and the relative industrialization of the municipality.

In order to provide a mammalian endpoint, the ability of sludge extracts to induce sister chromatid exchanges (SCE's) in human peripheral lymphocytes has been examined. It is quite difficult to culture the cells in the presence of the extracts and only partial results are currently available. We have discovered a direct-acting agent(s) in an acetone extract of Chicago sludge. A 100 µl sample of a 15 fold concentrated sample of this extract induced a statistically significant increase in the frequency of SCE's (p0.01). The control frequency was 0.21 $\pm$ 0.02 SCE/chromosome while the sample increased the frequency to 0.32 $\pm$ 0.02 SCE/chromosome. A positive control of 5 mM ethyl methane sulfonate (EMS) induced a SCE/chromosome frequency of 0.48 $\pm$ 0.02. This acetone extract is not directly mutagenic when tested with the Salmonella assay but also showed activation by mamalian S-9. The results also show that these acetone extracts are toxic to human lymphocytes at high concentrations. Further studies with this assay are in progress.

The in situ tests of sludge-amended soil using Chicago sludge and the maize wx locus assay demonstrate that mutagens are available to the plant when sludge is used as a soil amendment. Toxic as well as mutagenic responses were observed. The laboratory studies indicate that sludge can induce a substantial number of mutations at the wx locus in inbred Early-Early Synthetic as well as reverse mutation at the wx-90 locus observed in the field studies. Champaign sludge did not demonstrate mutagenic activity in the forward mutation assay with Early-Early Synthethic. The other sludges were not tested in this assay because of time constraints.. These studies indicate that mutagens present in sludge-amended soil are transported into a crop plant and can induce genetic damage in germ cells. However, these studies do not provide any indication of mutagenicity in the kernels grown on sludge-amended soil or transfer of the mutagens from the sludge to ground or surface waters. However, these results do suggest the need for further study of the possible adverse effects of land application of sludge from large industrialized cities.

SECTION 3

RECOMMENDATIONS

As a result of the research conducted during this project, the following recommendations are presented.

1. There is a need for further study of the presence or absence of mutagens in sewage sludge. In single grab samples of sludge from two highly industrialized cities, the presence of mutagenic agents was detected. A systematic investigation of similar cities should be made to identify the nature of the sources of mutagenic agents in such sludges so that appropriate control measures can be developed.

2. The separation and identification of the specific mutagenic agents should be undertaken. It may be possible to develop new or alter existing sewage treatment methods to deactivate these mutagens.

3. When sludge containing mutagenic agents is used as a soil amendment, the fate of the mutagens is unknown. In the field studies conducted, mutagens were found to be available to plants growing in these soils. Thus, the mutagens could percolate through the soil and be a potential ground water contaminant. On the other hand, they could be decomposed over time by soil microorganisms. It is recommended that the long term fate of the mutagens in sludge-amended soils be investigated through appropriate field and laboratory studies.

4. The use of multiple tests for mutagenicity is strongly recommended. In these studies the results are comparable using several different test systems although some differences were noted. Since each genetic assay can only detect a limited spectrum of genetic damage, it is important to be able to examine the results from different test systems with different genetic endpoints.

5. There is a need to examine if the mutagens in the soil can move through the food chain and reach human populations. Studies can be made to examine the effects of feeding grain grown on sludge-amended soil to test animals, to help determine if sludge application should be made to cropland.

SECTION 4

CHEMICAL FRACTIONATION

The initial studies were conducted on a sample of Chicago sewage sludge from the Southwest Treatment Plant of the Chicago Municipal Sanitary District at Stickney, Illinois. This sample was obtained from the storage tanks of the University of Illinois Agronomy Research Center at Elwood, Illinois where it was stored before being applied to the fields. This sample was a composite of the sludge received at Elwood during the period from November 1978 and July 1979. Sixteen liters of sewage sludge were centrifuged at 12,500 rpm in a Sorvall GSA rotor (max 25400 x g) for 60 minutes. One liter of the supernatant liquid was filter-sterilized by passing through a 0.45 μm Millipore filter (fraction A1). Another two liters of supernatant liquid were passed through a column of XAD-2 resin previously cleaned as recommended by Junk et al (7). The column was eluted with 200 ml of methanol (fraction A2a), and then with 200 ml of diethylether (fraction A2b). Two milliliters of dimethylsulfoxide(DMSO) were added to each eluate. The eluates were reduced in volume on a rotovac until only the DMSO solution remained. In addition, ten liters of supernatant were concentrated 100-fold by vacuum distillation at 30°C (fraction A3). Each of these samples was tested for mutagenicity in the TA98 and TA100 strains with and without mammalian microsomal activation except for fraction A3. Fraction A3 was highly contaminated with bacteria and could not be filter sterilized because of its high viscosity.

The 2.5 kg solid pellet was extracted three times with two volumes of acetone and the extracts were pooled (fraction E1). The residue was extracted three times with one volume of n-hexane and these extracts were pooled (fraction E2). Fifteen milliliters of DMSO were added to both the acetone and hexane extracts and each extract was concentrated as before. It was necessary to lyophilize fraction E1 to remove the residual water sequestered from the solid by the acetone. These solutions were filter sterilized and assayed for mutagenicity as described above.

In these procedures and the modifications described below, there are several steps that could result in the loss of volatile compounds. However, there would also be substantial loss of volatiles from any sludge applied to soils and the mutagenicity of the nonvolatile fractions are of greater concern for sludge disposal than the volatile

compounds. The protocols used in this study will permit the separation and identification of mutagenic compounds that would be mixed into the soil and may be subsequently incorporated into crops, removed by runoff or leached through the soil to groundwater sources.

To determine the amount of mutagenic activity recovered by the acetone extraction, another 100 g of pellet obtained from a different subsample of sludge were extracted three times with 200 ml of acetone. The extracts were tested separately as fractions F1, F2, and F3. The weight of material dissolved in each fraction was measured by evaporation of 200 µl and 500 µl aliquots to dryness in a desiccator. The residue remaining was weighed and the concentration of acetone-soluble material in each fraction was calculated. These results are presented in Table 1. There is increasing dissolution of the sludge pellet in the sequential extractions. The initial extraction also removes most of the sequestered water that could not be removed by centrifugation. Thus, the increasing solubility of the sludge may reflect the decreasing quantity of residual water content in the solids. Fifty milliliters of each fraction were dried over anhydrous sodium sulfate. Five milliliters of DMSO were added and each fraction was concentrated as before.

Additional single grab samples were obtained from municipal treatment plants in Champaign(CHMP), Hinsdale(HNSD), Kankakee(KANK), and Sauget(SAUG). The Chicago(CHI) sample described above was also studied further. The treatment plants for Champaign, Hinsdale, and Kankakee primarily or exclusively treat household wastes. The Sauget plant treats the effluent from a large, synthetic organic chemical manufacturing plant in addition to domestic sewage. Additional information regarding these sludge samples is provided in Table 2.

The protocol for extracting the sludge was altered in order that the crude extracts could be fractionated in better defined chemical classes. This protocol is outlined in Figure 1.

Chloroform:methanol was choosen because it is considerably less polar than acetone although more polar than hexane. This modification has advantages in that it permits further chemical fractionation of the crude extracts and it reduces the amount of water sequestered during the extraction of the wet solids. The sequestered water is eliminated from these extracts by extracting lyophilized solids rather than the wet solids originally employed. The yields of residue obtained from the C:M extraction of these five sludge samples are also presented in Table 3. These yields are similar to those obtained by the acetone extraction.

TABLE 1. CONCENTRATION OF ACETONE SOLUBLE MATERIAL AND WATER CONTENT OF THE SEQUENTIAL CHICAGO SLUDGE EXTRACTS

Eluate	Concentration of Soluble Material (mg/ml)	Water Content (% Volume)
F1	5.5	10
F2	7.5	4
F3	24.3	0.5

TABLE 2. GENERAL INFORMATION ON THE MUNICIPAL SEWAGE SLUDGE SAMPLES TO BE EXAMINED FOR MUTAGENICITY

Municipality Sampled	Sampling Date	Source Type	Treatment Process	Nature of Sample	% Dry Solids (wt/wt)
Chicago	10/79	ind/resid	Anaerobic	Slurry	3.5
Champaign	11/80	residential	Aerobic	Slurry	4.0
Kankankee	4/81	ind/resid	Aerobic/ Anaerobic	Slurry	3.5
Hinsdale	4/81	?/resid	Aerobic	Solid	—
Sauget	7/81	ind/resid	Aerobic	Slurry	13.9

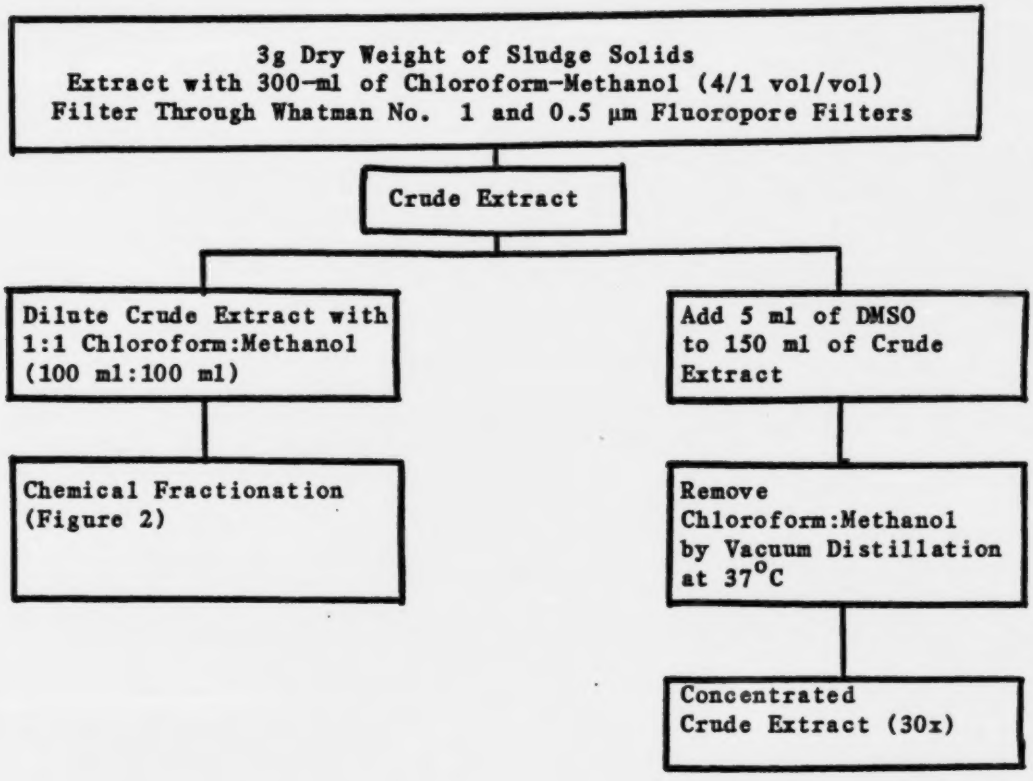
3g Dry Weight of Sludge Solids
Extract with 300-ml of Chloroform-Methanol (4/1 vol/vol)
Filter Through Whatman No. 1 and 0.5 µm Fluoropore Filters
Crude Extract
Dilute Crude Extract with 1:1 Chloroform:Methanol (100 ml:100 ml)
Chemical Fractionation (Figure 2)
Add 5 ml of DMSO to 150 ml of Crude Extract
Remove Chloroform:Methanol by Vacuum Distillation at 37°C
Concentrated Crude Extract (30x)

Figure 1. Outline of the Original Extraction Protocol

TABLE 3. YIELDS OF ACETONE AND CHLOROFORM:METHANOL SOLUBLE MATERIAL EXTRACTED FROM MUNICIPAL SEWAGE SLUDGES

Solvent	Sample	% Dry Solids	Mass of Pelleted Solids (g)	Mass of Dry Solids (g)	Mass of Residue (mg)	Mass of Residue per Mass of Solids (mg/g)	% Recovery
Acetone	CHMP	4.0	500	20.0	1540	77.0	7.7
	CHI	3.5	500	17.5	1386	79.2	7.9
C:M	CHMP	4.0		3.0	194	64.8	6.5
	CHI	3.5		3.0	232	77.3	7.7
	HNSD	—		3.0	140	46.7	4.7
	KANK	3.5		3.0	374	124.7	12.5
	SAUG	13.9		3.0	417	139.0	13.9

These crude extracts were then subjected to a further fractionation as outlined in Figure 2. These additional separation steps are necessary in order to determine the presence or absence of mutagenic activity and minimize synergistic and antagonistic effects of other species potentially present in these complex mixtures. This procedure separates the extracts into four general chemical classes: strongly acidic, weakly acidic, basic, and neutral compounds. The yields for each of these fractions are given in Table 4. These yields are needed to permit the calculation of the level of mutagenic activity per unit mass of material being tested. The Kankakee sludge did not separate well as it formed emulsions that could not be broken. Therefore, the testing of individual fractions of the Kankakee sludge was not possible. The data in Table 4 have been placed in the order of the highest to the lowest yields of extracted mass. The majority of the mass in each case was in the neutral fraction (63-92%). The other classes show the following ranges: basic (1.67-25%), weakly acidic (3.5-12%), and strongly acidic (2.9-6.3%). These various extracts and fractions were then available to be examined for mutagenic activity by several test systems described in subsequent sections of this report.

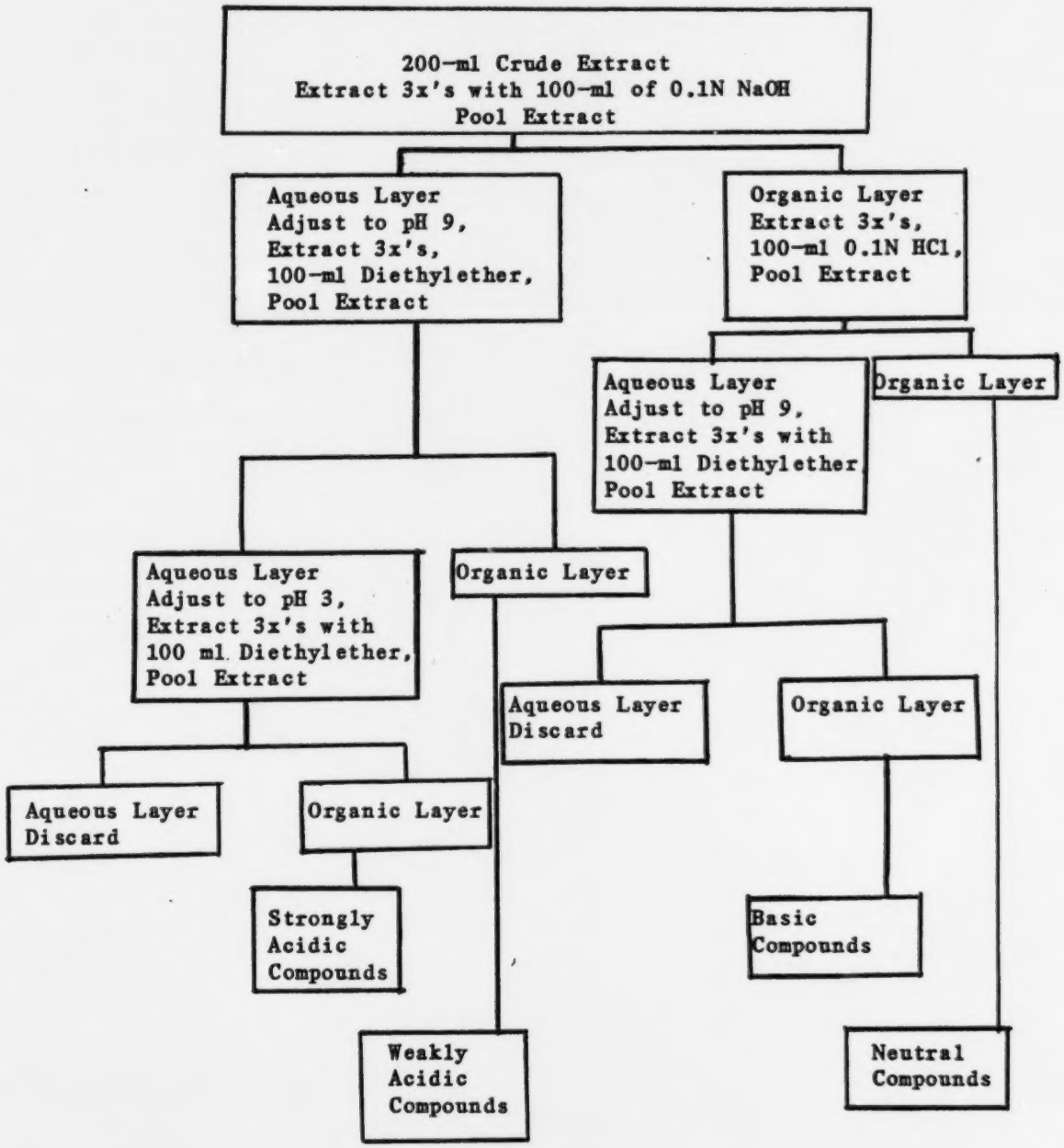

Figure 2. Schematic Outline of the Modified Fractionation Protocol

TABLE 4. YIELDS OF THE VARIOUS CHEMICAL FRACTIONS OBTAINED FROM THE CHLOROFORM:METHANOL EXTRACTION OF MUNICIPAL SEWAGE SLUDGES

Sample	Fraction	Conc (µg/ml)	Total Vol of Fraction (ml)	Total Amount of Residue (mg)	% of Total
CHMP	Neut	199	300	59.8	23.0
	Basic	79.5	300	23.8	9.2
	W. Acid	38.2	300	11.5	4.4
	S. Acid	—	300	164.8	63.4
CHI	Neut	294	300	88.3	73.8
	Basic	53.2	300	15.9	13.3
	W. Acids	26.4	300	7.9	6.6
	S. Acids	25.0	300	7.5	22.5
HNSD	Neut	1020	300	305	92
	Basic	17.9	300	5.4	1.6
	W. Acid	38.2	300	11.5	3.5
	S. Acid	31.6	300	9.6	2.9
SAUG	Neut	525	300	158	83.4
	Basic	19.4	300	5.8	3.1
	W. Acid	51.8	300	15.5	8.2
	S. Acid	33.6	300	10.1	5.3

SECTION 5

MICROBIAL ASSAYS

METHODS AND MATERIALS

The Salmonella/microsome reverse-mutation assay employing Ames tester strains TA98 and TA100 was conducted using the top agar incorporation procedure described by Ames et al (8), incorporating the recommendations of deSerres and Shelby (9). TA98 and TA100 are the most widely used strains because together they are the most sensitive and they respond to the widest range of chemical types. The overnight cultures had densities of approximately 2×10^9 cells/ml and the spontaneous reversion frequencies of each strain were within their acceptable range (9). The strains were periodically tested for their genetic markers as prescribed by Ames et al (8). Positive controls for the S-9 activated samples were run using benzo(a)pyrene (BaP). The slopes of the linear dose-response curves in revertants per µg of BaP averaged 221 and 344 for TA98 and TA100, respectively. The hepatic S-9 fraction was prepared as described by Ames et al (8) from male Sprague-Dawley rats induced with either Aroclor 1254 or phenobarbitol. The purpose of adding S-9 to the assay is to simulate mammalian metabolism of test compounds, and, in particular, to detect promutagens, compounds that become mutagenically active as a result of metabolism. There is a wide range of response of the Ames strains to a given quantity of mutagenic compound. As shown by Johnston and Hopke (10), the response per unit weight varies over 6 orders of magnitude so that a small response may be the result of a large quantity of a weak mutagen or a small quantity of a potent agent. Also, a weak response is not an indication that a sample is harmless. There are potent carcinogens such as the nitrosamines that show low activity in the Ames strains. This difference in sensitivity emphasizes the need for a multiorganism testing approach as used in this study. The lack of a quantitative relationship between the mutagenic activity in the Ames assay and carcinogenic potency in animal cancer bioassays is also widely recognized as indicated by the use of the Ames strains as the first tier of genetic toxicology tests recommended by the U.S. Environmental Protection Agency (11). It recognizes the excellent qualitative response of the Ames assay to mutagens and this screening is used to qualitatively separate chemicals needing further examination in other genetic assays. The Ames strains are relevant and useful in evaluating the possible presence of mutagenic activity in complex environmental mixtures, but it does not tell whether a sample is a threat to humans. That relationship requires identification of the active components and carcinogenicity testing.

RESULTS

The mutagenic activities of the aqueous supernatent liquids are shown in Table 5. Fractions A1 and A2b did not show the presence of any direct-acting mutagens. Fraction A2a evoked equivocal responses in strain TA98 with Aroclor/S-9 activation and in TA100 without activation. Therefore, no appreciable mutagenic activity was associated with the aqueous supernatant liquid.

The examination of the extracts of the sludge pellet are also presented in Table 5. Direct acting mutagens were not detected in extracts E1 or E2 (data not shown). With the addition of the Aroclor/S-9 mix, these fractions became mutagenic. The equivalent of 0.6 µl of E1 produced an approximate doubling in the number of revertant colonies per plate. The unusual dose-response behavior of extract E2 should be noted. The elevated but flat response of the assay to this fraction precluded estimation of a reliable doubling dose. This dose-response behavior illustrates the difficulties of analyzing complex mixtures and indicates the need for further fractionation to resolve the active compounds. The equivalent amount of original sludge required to double the number of TA98 colonies per plate is 155 µl for fraction E1.

The successive extraction of mutagenic compound(s) by acetone from the sludge pellet is presented in Table 6. The dose-response curves for the three fractions assayed with TA98 and TA100 are shown in Figures 3 and 4, respectively. The response of an organism to a pure mutagenic compound would follow a curve where the number of mutational events observed increases with increasing concentration to the point where on the average one lethal mutation per organism is occurring. From that point there will be a decline in response due to the accumulation of deleterious mutations leading to the killing of the test organism. In complex mixtures such as these extracts, there can also be direct toxicity to the organism superimposed on the mutagenic response curve leading to declining events with increasing concentration at a lower applied dose. As reflected by the initial slopes in Figures 3 and 4, the mutagenic activity recovered in fractions F1 and F2 are roughly the same for both TA98 and TA100. The mutagenic activity present in F3 was less than in fractions F1 and F2. When assayed with TA100, fractions F1 and F2 are more toxic per volume of extract than fraction F3 since there are lower numbers of revertant colonies observed for the higher doses assayed. It, therefore, appears that the majority of the mutagenic activity was recovered in the first two extractions of the pellet. The third extraction did not increase the recovery of these mutagenic substances. Although additional mutagenic activity was extracted, there was a decrease in the mutagenicity of per unit weight of extracted material. However, the amount of the pellet dissolved by the acetone increased in each successive extraction as shown in Tables 1 and 6. Therefore, the amount of mutagenic activity per mg of acetone-soluble material decreased with each extraction as shown in Figures 5 and 6 for TA98 and TA100, respectively. It is apparent that fraction F1 had the highest mutagenicity to mass ratio of the three fractions.

TABLE 5. MUTAGENICITY OF POOLED EXTRACTS OF CHICAGO SEWAGE SLUDGE USING AMES STRAINS TA98 AND TA100

Sample	Sludge Extracts: Volume per Plate (µl)	Dilution Factor	Equivalent Volume per Plate (µl)	Concentration Factor (V/V)	Equivalent Vol. of Neat Sludge per Plate (µl)	Revertants per Plate (Mean ± Standard Deviation): TA98 -S9	TA98 +S9	TA100 -S9	TA100 +S9
DMSO	100						57 ± 23[a]		
E1	10	0.02	0.2	325.	65		89 ± 17		
	20	0.02	0.4		130		100 ± 15		
	30	0.02	0.6		195		131 ± 17		
	40	0.02	0.8		260		133 ± 19		
	50	0.02	1.0		325		136 ± 21		
E2	10	0.1	1.0	260.	260		89 ± 24		
	20	0.1	2.0		520		103 ± 25		
	30	0.1	3.0		780		109 ± 21		
	50	0.1	5.0		1300		117 ± 29		
	100	0.1	10.0		2600		100 ± 12		
Water	2000					60 ± 5[b]	80 ± 6	155 ± 3	184 ± 8
A1	100		100.	1.25	125	69 ± 7	86 ± 10	165 ± 10	192 ± 8
	300	1.0	300.		375	63 ± 1	90 ± 2	167 ± 18	192 ± 4
	2000	1.0	2000.		2500	67 ± 20	79 ± 6	161 ± 4	174 ± 2
DMSO	100					31 ± 1	70 ± 21	115 ± 7	130 ± 7
A2a	50	0.1	5.	125.	625	37 ± 1	72 ± 11	131 ± 16	103 ± 10
	100	0.1	10.		1250	29 ± 1	59 ± 13	143 ± 17	114 ± 4
	20	1.0	20.		2500	35 ± 8	55 ± 9	146 ±	140 ± 17
	50	1.0	50.		6250	39 ± 0	60 ± 23	140 ± 40	146 ± 2
	100	1.0	100.		12500	37 ± -	95 ± 45	173 ± 8	154 ± 6
A2b	50	0.1	5.	125.	625	29 ± 2	44 ± 15	115 ± 9	108 ± 13
	100	0.1	10.		1250	35 ± 1	50 ± 3	139 ± 24	125 ± 25
	20	1.0	20.		2500	42 ± 5	55 ± 13	127 ± 18	135 ± 7
	50	1.0	50.		6250	36 ± 6	nd	156 ± 16	153 ± 17
	100	1.0	100.		12500	41 ± 1	nd	146 ± 12	148 ± 17

[a] data are derived from triplicate platings
[b] data are derived from duplicate platings

TABLE 6. MUTAGENICITY OF THE THREE SEQUENTIAL ACETONE EXTRACTIONS OF CHICAGO SEWAGE SLUDGE IN AMES STRAINS TA98 AND TA100 FOLLOWING AROCLOR/S-9 ACTIVATION

Sample	Volume per Plate(µl)	Dilution Factor	Equivalent Volume per Plate(µl)	Concentration (mg/ml)	Mass per Plate(mg)	TA98 Revertants per Plate	TA100 Revertants per Plate
Control	100			0.	0.	52 ± 6	130 ± 7
F1	50	1	50	2.1	0.1	76 ± 6	162 ± 12
	100	1	100	2.1	0.2	97 ± 9	218 ± 11
	20	10	200	2.1	0.4	160 ± 11	254 ± 1
	50	10	500	2.1	1.0	225 ± 28	280 ± 3
	100	10	1000	2.1	2.1	nd	68 ± 23
F2	50	1	50	7.3	0.4	83 ± 1	164 ± 4
	100	1	100	7.3	0.7	110 ± 20	235 ± 24
	20	10	200	7.3	1.5	144 ± 30	284 ± 1
	50	10	500	7.3	3.7	164 ± 7	297 ± 20
	100	10	1000	7.3	7.3	nd	172 ± 13
F3	50	1	50	23.9	1.2	57 ± 6	166 ± 18
	100	1	100	23.9	2.4	78 ± 9	171 ± 13
	20	10	200	23.9	4.8	93 ± 11	208 ± 14
	50	10	500	23.9	12.0	124 ± 13	253 ± 11
	100	10	1000	23.9	23.9	nd	287 ± 35

nd: indicates that a value was not determined.

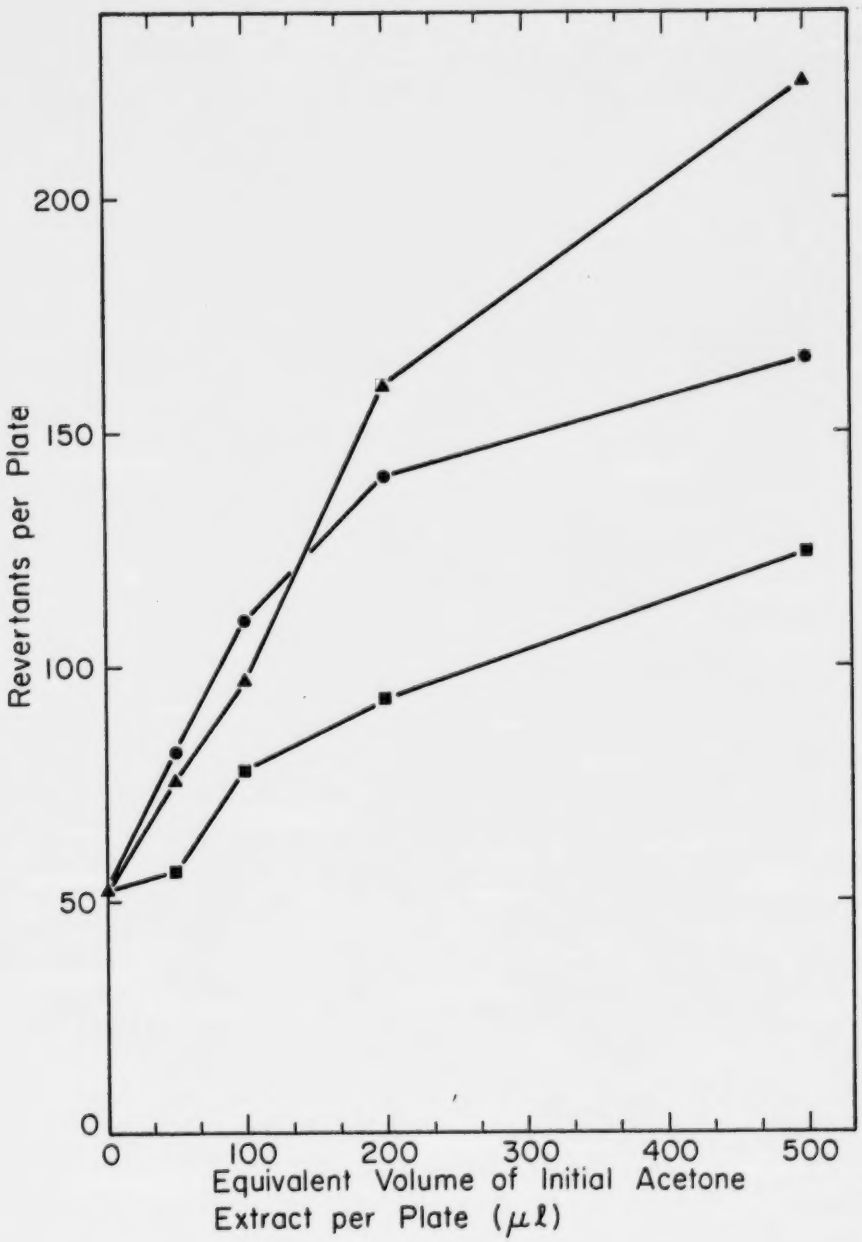

Figure 3. Mutagenicity in strain TA98 on an equivalent volume of acetone extract basis for the three sequential extractions of the sewage sludge pellet following activation with S-9: first extraction (F1) ▲, second extraction (F2) ●, third extraction (F3) ■

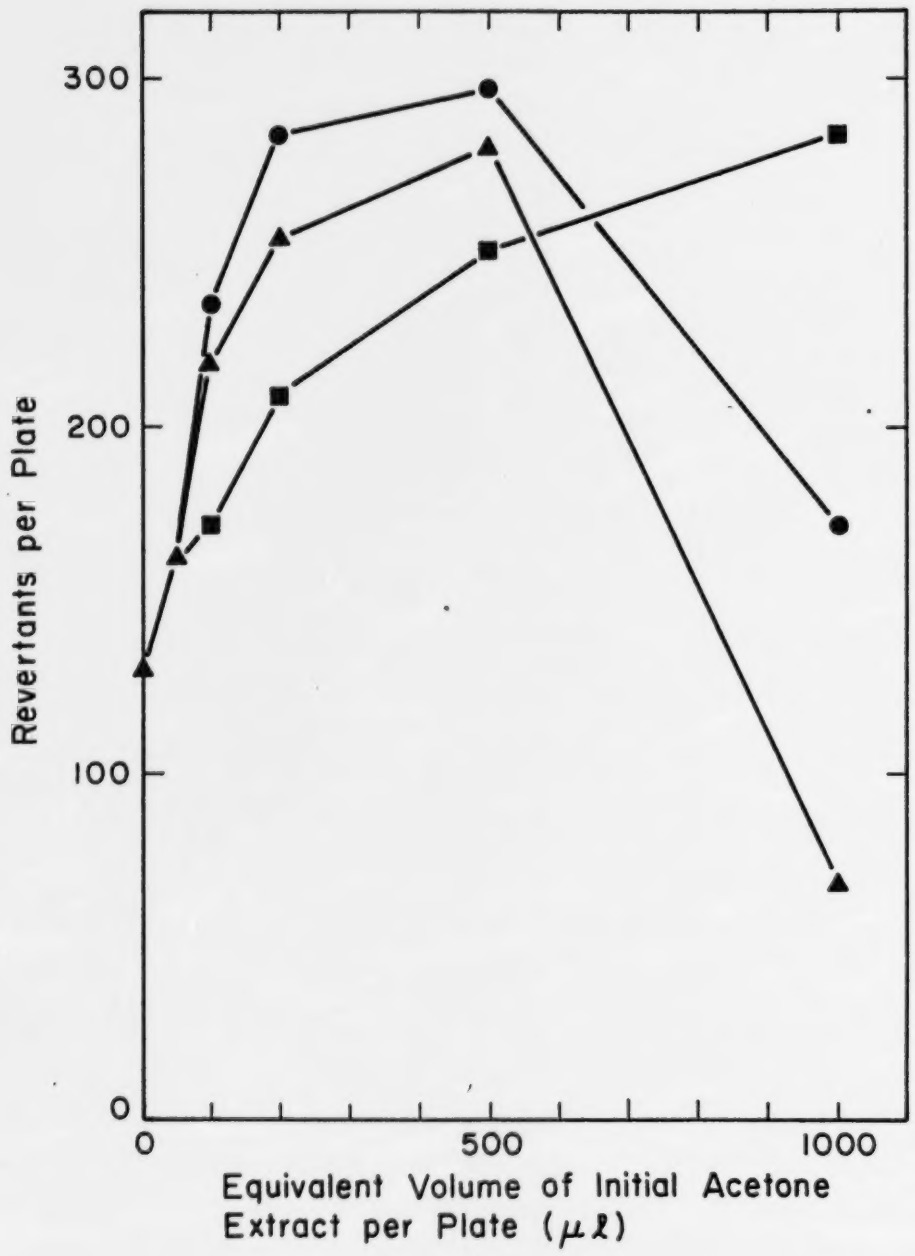

Figure 4. Mutagenicity in strain TA100 on an equivalent volume of acetone extract basis for the three sequential extractions of the sewage sludge pellet following activation with S-9: first extraction (F1) ▲, second extraction (F2) ●, third extraction (F3) ■

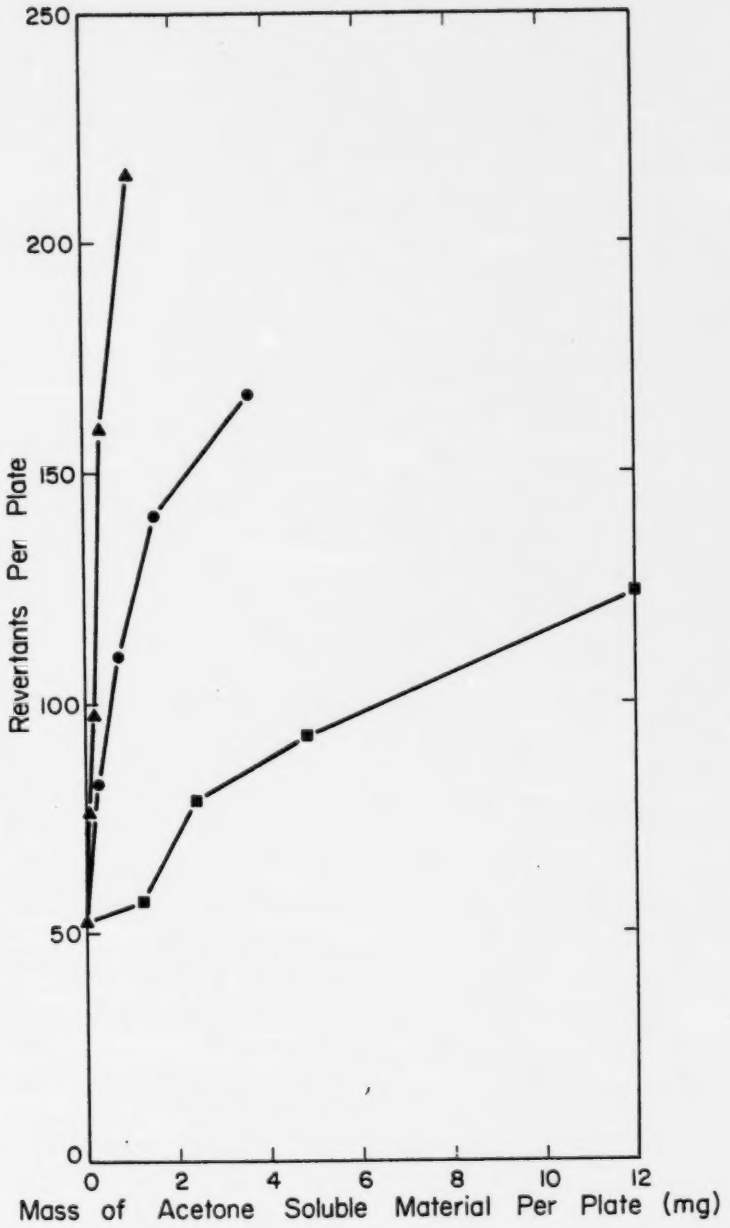

Figure 5. Mutagenic response in strain TA98 against the mass of acetone-soluble material from the three sequential extractions of the sludge pellet following activation with S-9: first extraction (F1) ▲ , second extraction (F2) ● , third extraction (F3) ■

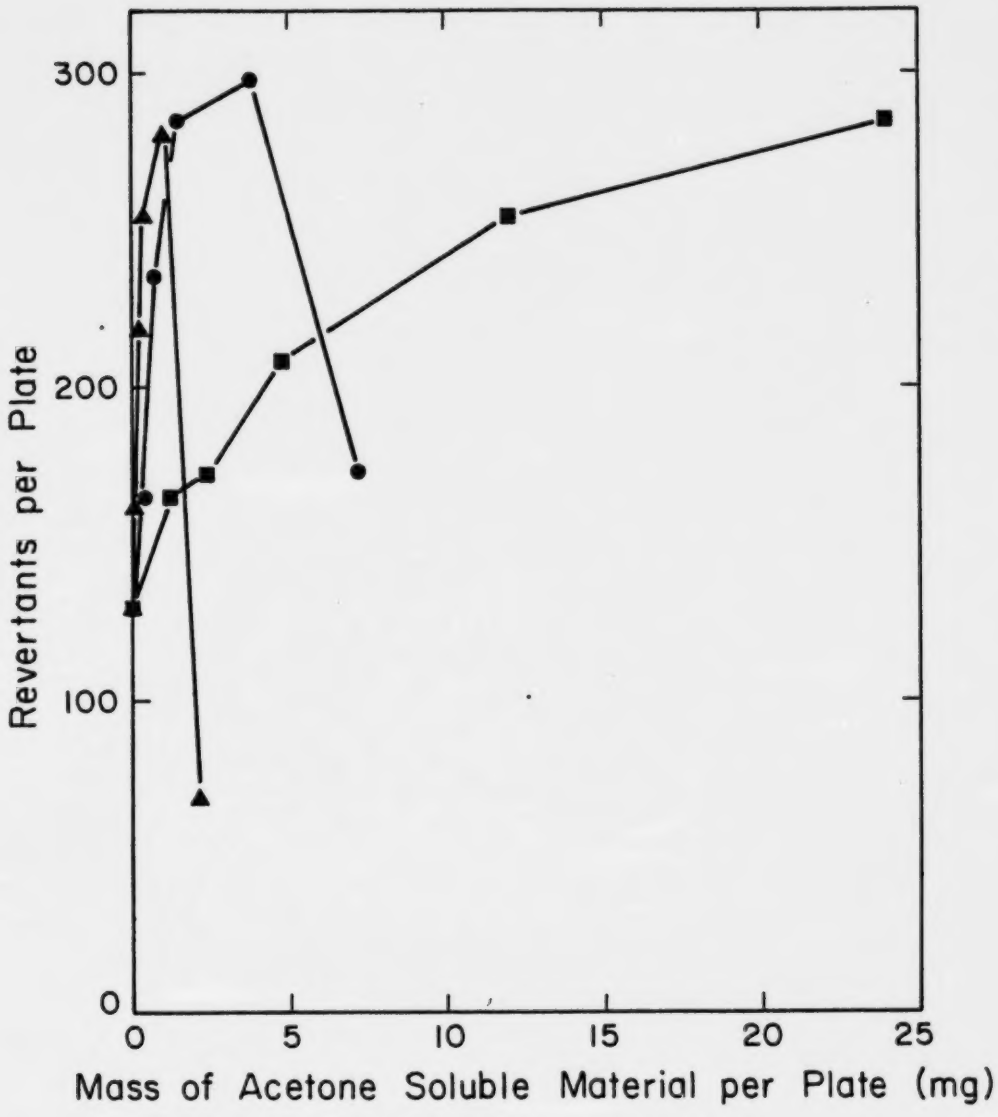

Figure 6. Mutagenic response in strain TA100 against the mass of acetone-soluble material from the three sequential extractions of the sludge pellet following activation with S-9: first extraction (F1) ▲, second extraction (F2) ● , third extraction (F3) ■

Subsequently, the crude extracts from all five samples, Champaign, Chicago, Hinsdale, Kankakee, and Sauget, were examined for mutation induction. The data are presented in Table 7. The Sauget crude extract is very mutagenic. A doubling over the control in the number of revertants per plate was induced by 139 μg per plate of Sauget crude extract residue in the absence and presence of Aroclor/S-9. Both in the absence and presence of Aroclor/S-9 linear dose-dependent reponses are induced by the Sauget crude extract (Figure 7). The slopes of the responses indicates that the Sauget crude extract residue induced 260 revertants per mg without S-9 and 780 revertants per mg with Aroclor/S-9. The presence of Aroclor/S-9 enhanced the yield of revertants per mg of residue by a factor of three over its absence.

The Champaign, Chicago, Hinsdale, and Kankakee crude extracts did not induce either a doubling over the control or a dose-dependent increase in the number of revertants per plate. Therefore, these crude extracts were not directly mutagenic. Similarly, these extracts demonstrated a negative response with the addition of either type of S-9.

The results of the mutation assays using in TA98 of each subfraction are presented in Table 8. The concentration of each fraction are quite low. Thus, fractions were tested at the lower limits of resolution. The slopes of the dose-dependent response were determined for only those fractions that demonstrated: 1) doubling over the control in the number in revertants per plate, 2) dose-dependent response which exhibit a minimum of two doses that equaled or exceeded a doubling over the control.

All the Sauget fractions induced a mutagenic response in TA98 (Table 8). The dose-dependent response induced by the Sauget fractions in the absence or presence of Aroclor/S-9 are shown in Figures 8-15. The basic fraction was the most mutagenic (1600 revertants per mg of residue) and the weakly acidic was the least mutagenic (27.6 revertant/mg residue). Both the neutral and strongly acidic fractions were very mutagenic, 129 and 380 revertants/mg residue, respectively.

As shown in Table 9, the Sauget sample has substantially different properties than any of the other samples. With Aroclor/S-9, the basic fraction was the most mutagenic. However, the Aroclor/S-9 eliminated the mutagenic response exhibited by the weakly acidic fraction (Figure 14). Thus, both the basic and weakly acidic fractions exhibited a decreased yield of revertants per mg residue demonstrating a net metabolic deactivation of mutagens into non-mutagens. The neutral and strongly acidic fractions were very mutagenic and exhibited an enhanced yield of revertants with Aroclor/S-9. With Aroclor/S-9, the neutral fraction induced 248 revertants per mg residue whereas without Aroclor/S-9, this fraction induced 129 revertants per mg residue. The mutagenic activity of the neutral fraction was enhanced 1.9 fold (248 revertants per mg residue/129 revertants per mg residue) by Aroclor/S-9. Similarly, the strongly acidic fraction exhibited an enhanced mutagenic

TABLE 7. MUTAGENICITY OF CRUDE CHLOROFORM:METHANOL EXTRACTS IN TA98 IN THE ABSENCE AND PRESENCE OF S-9

Sample	Vol (µl)	Conc Factor	Equiv Vol (µl)	Conc (mg/ml)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9[a]	+S-9[b]
CHMP	100	-	100	0	0.0	25	60	32
	100	0.1	10	650	6.5	31	50	40
		1.0	100		65.0	25	50	48
		10.0	1000		650.0	36	29	31
		30.0	3000		1900.0	K[c]	K	34
CHI	100	-	100	0	0.0	21	58	40
	100	0.1	10	770	7.7	22	55	32
		1.0	100		77.0	18	58	39
		10.0	1000		770.0	26	35	65
		30.0	3000		2300.0	K	18	42
HNSD	100	-	100	0	0.0	27	71	40
	100	0.1	10	470	4.7	25	71	47
		1.0	100		47.0	36	68	55
		10.0	1000		470.0	45	74	57
		30.0	3000		1400.0	41	64	63
KANK	100	-	100	0	0.0	23	61	
	100	0.1	10	1250	12.5	25	56	
		1.0	100		125.0	29	42	
		10.0	1000		1250.0	27	38	
		30.0	3000		3750.0	K	K	
SAUG	100	-	100	0	0.0	28	66	
	100	0.1	10	1390	13.9	30	79	
		1.0	100		139.0	61	214	
		10.0	1000		1390.0	377	741	
		30.0	3000		4170.0	1137	3363	

[a] Aroclor/S-9
[b] phenobarbital/S-9
[c] K: absence or partial absence of lawn

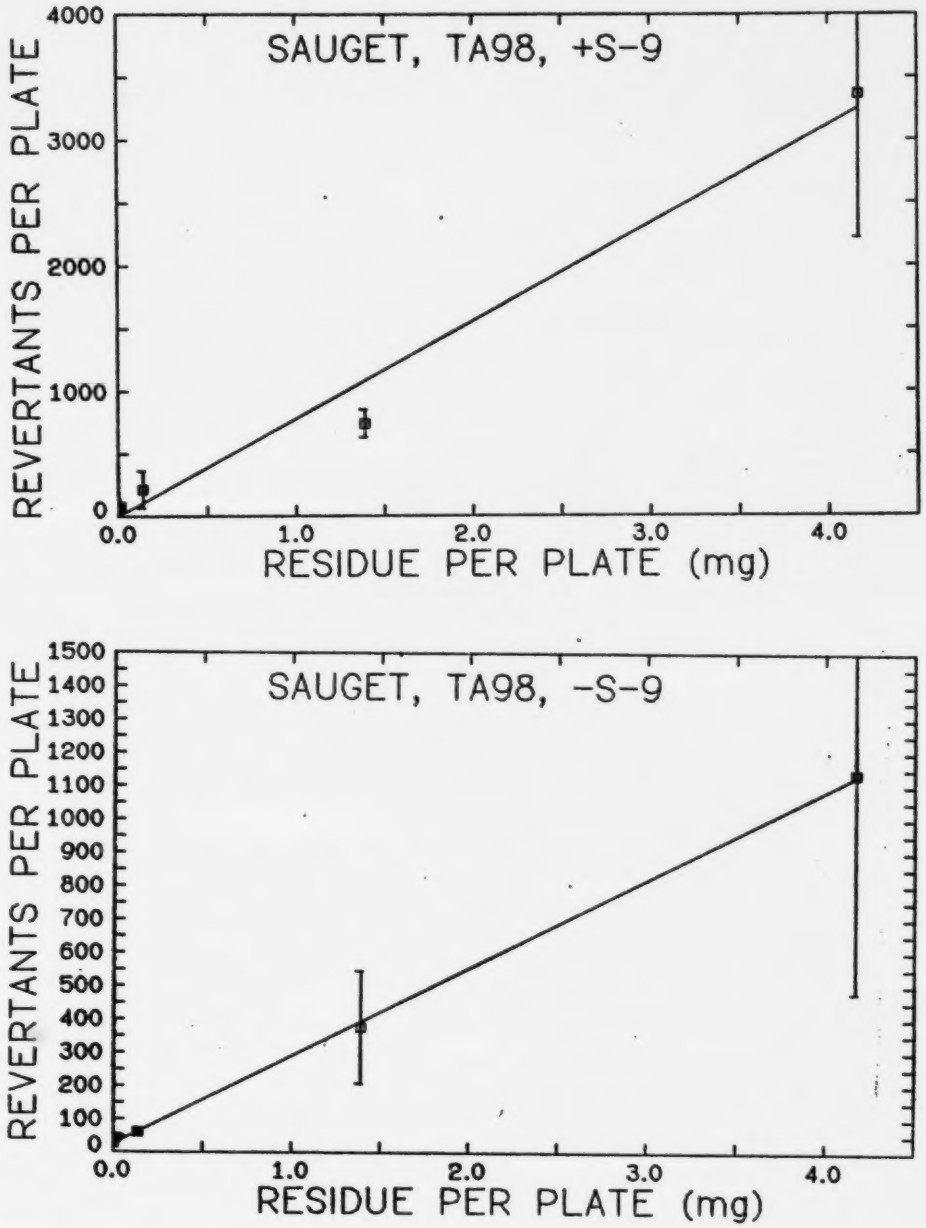

Figure 7. Dose-response curves for Sauget crude chloroform:methanol extracts in strain TA98 with (top) and without (bottom) S-9 activation.

TABLE 8. MUTAGENICITY OF MUNICIPAL SEWAGE SLUDGE FRACTIONS IN TA98 WITH AND WITHOUT S-9

Sample	Vol (µl)	Conc Factor	Equiv Vol (µl)	Conc (mg/ml)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9[a]	+S-9[b]
CHMP	100	0	0	0	0	24	53	32
Neutr	50	3.0	50	199	29.9	29	34	36
	100	3.0	300		59.7	26	52	32
	50	30.0	1500		299.0	27	48	39
	100	30.0	3000		597.0	19	39	41
Bases	100	0	0	0	0	24	53	32
	50	3.0	150	79.5	11.9	25	47	54
	100	3.0	300		23.9	34	54	59
	50	30.0	1500		119.0	64	51	50
	100	30.0	3000		239.0	97	99	69
Weak	100	0	0	0	0	24	53	32
Acids	50	3.0	150	38.2	5.7	27	58	40
	100	3.0	300		11.5	40	54	43
	50	30.0	1500		57.3	50	88	67
	100	30.0	3000		115.0	60	88	71
Strong	100	0	0	0	0	24	53	32
Acids	50	3.0	150	550	8.2	32	44	40
	100	3.0	300		16.5	34	58	41
	50	30.0	1500		82.0	71	109	72
	100	30.0	3000		165.0	116	119	100

TABLE 8. (CONTINUED)

Sample	Vol (µl)	Conc Factor	Equiv Vol (µl)	Conc (mg/ml)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9	+S-9
CHI	100	0	0	0	0	16	49	40
neutr	50	3.0	150	294	44.0	19	57	39
	100	3.0	300		88.0	17	64	45
	50	30.0	1500		441.0	26	60	37
	100	30.0	3000		882.0	14	53	36
bases	100	0	0	0	0	16	49	40
	50	3.0	150	53.2	8.0	23	63	22
	100	3.0	300		16.0	26	59	34
	50	30.0	1500		80.0	66	70	40
	100	30.0	3000		160.0	58	76	40
weak	100	0	0	0	0	16	49	40
acids	50	3.0	150	26.4	3.0	22	65	36
	100	3.0	300		6.1	15	87	41
	50	30.0	1500		30.0	38	66	41
	100	30.0	3000		61.0	38	66	41
str	100	0	0	0	0	16	49	40
acids	50	3.0	150	25.0	3.8	20	64	31
	100	3.0	300		7.6	25	67	37
	50	30.0	1500		38.0	49	94	36
	100	30.0	3000		76.0	53	99	63

TABLE 8. (CONTINUED)

Sample	Vol (µl)	Conc Factor	Equiv Vol (µl)	Conc (mg/ml)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9	+S-9
HNSD	100	0	0	0	0	32	85	40
neutr	50	3.0	150	1020	153.0	30	93	47
	100	3.0	300		360.0	31	100	36
	50	30.0	1500		1530.0		116	43
	100	30.0	3000		3600.0	39	106	60
bases	100	0	0	0	0	32	85	40
	50	3.0	150	17.9	2.7	31	95	32
	100	3.0	300		5.4	31	93	35
	50	30.0	1500		27.0	58	93	43
	100	30.0	3000		52.0	58	103	65
weak	100	0	0	0	0	32	85	40
acids	50	3.0	150	38.2	4.7	24	84	39
	100	3.0	300		9.4	36	90	39
	50	30.0	1500		47.0	82	105	66
	100	30.0	3000		94.0	79	113	67
str	100	0	0	0	0	32	85	40
acids	50	3.0	150	31.6	4.8	32	83	41
	100	3.0	300		9.6	32	72	49
	50	30.0	1500		48.0	69	97	67
	100	30.0	3000		96.0	94	112	—

TABLE 8. (CONTINUED)

Sample	Vol (µl)	Conc Factor	Equiv Vol (µl)	Conc (mg/ml)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9	+S-9
SAUG	100	0	0	0	0	31	66	
neutr	50	3.0	150	525	7.8	31	66	
	100	30.0	100		158.0	84	214	
	50	30.0	1500		788.0	145	293	
	100	30.0	3000		1580.0	253	488	
bases	100	0	0	0	0	31	66	
	50	3.0	150	19.7	2.9	37	76	
	100	3.0	300		5.8	49	68	
	50	30.0	1500		29.0	78	80	
	100	30.0	3000		58.0	127	130	
weak	100	0	0	0	0	31	66	
acids	50	3.0	150	51.8	7.8	31	66	
	100	3.0	300		15.5	39	61	
	50	30.0	1500		780.0	52	50	
	100	30.0	3000		1550.0	77	76	
str	100	0	0	0	0	31	66	
acids	50	3.0	150	33.6	5.0	49	55	
	100	3.0	300		30.0	30	59	
	50	30.0	1500		50.0	57	78	
	100	30.0	3000		301.0	73	101	

[a] Aroclor/S-9
[b] Phenobarbital/S-9

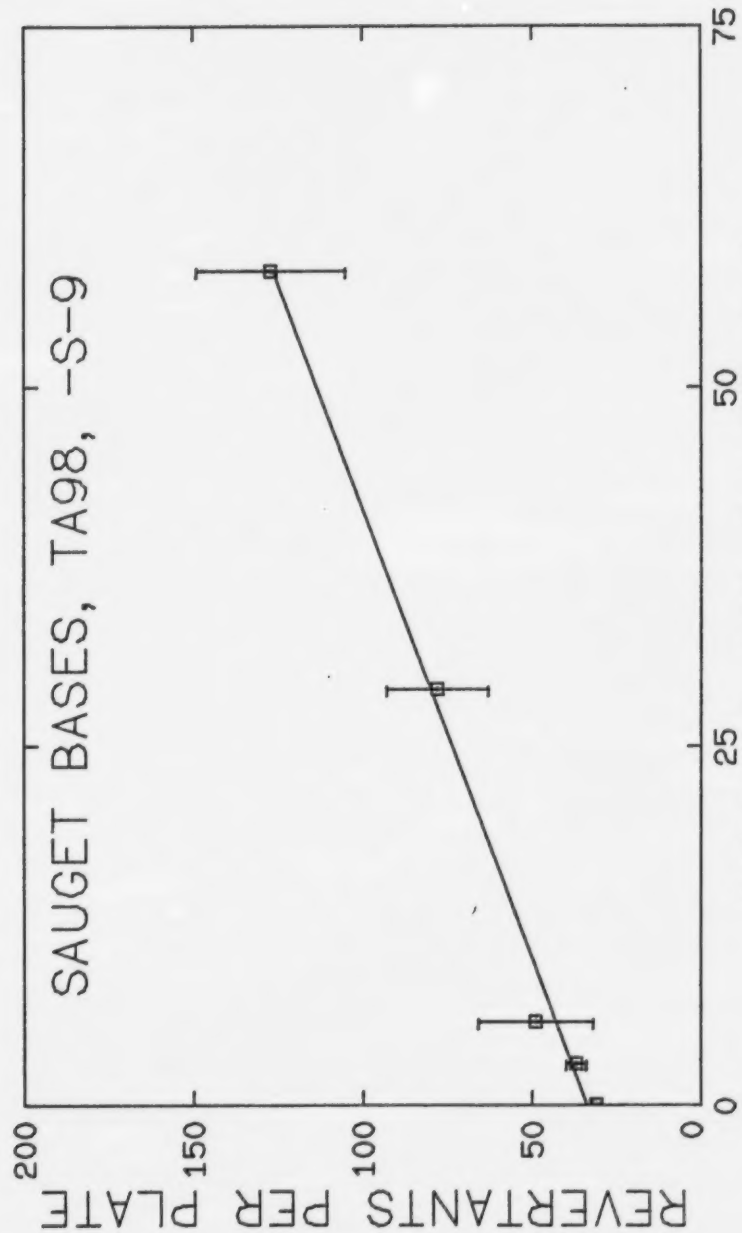

Figure 8. Dose-response curve for Sauget basic fraction without S-9 activation in strain TA98.

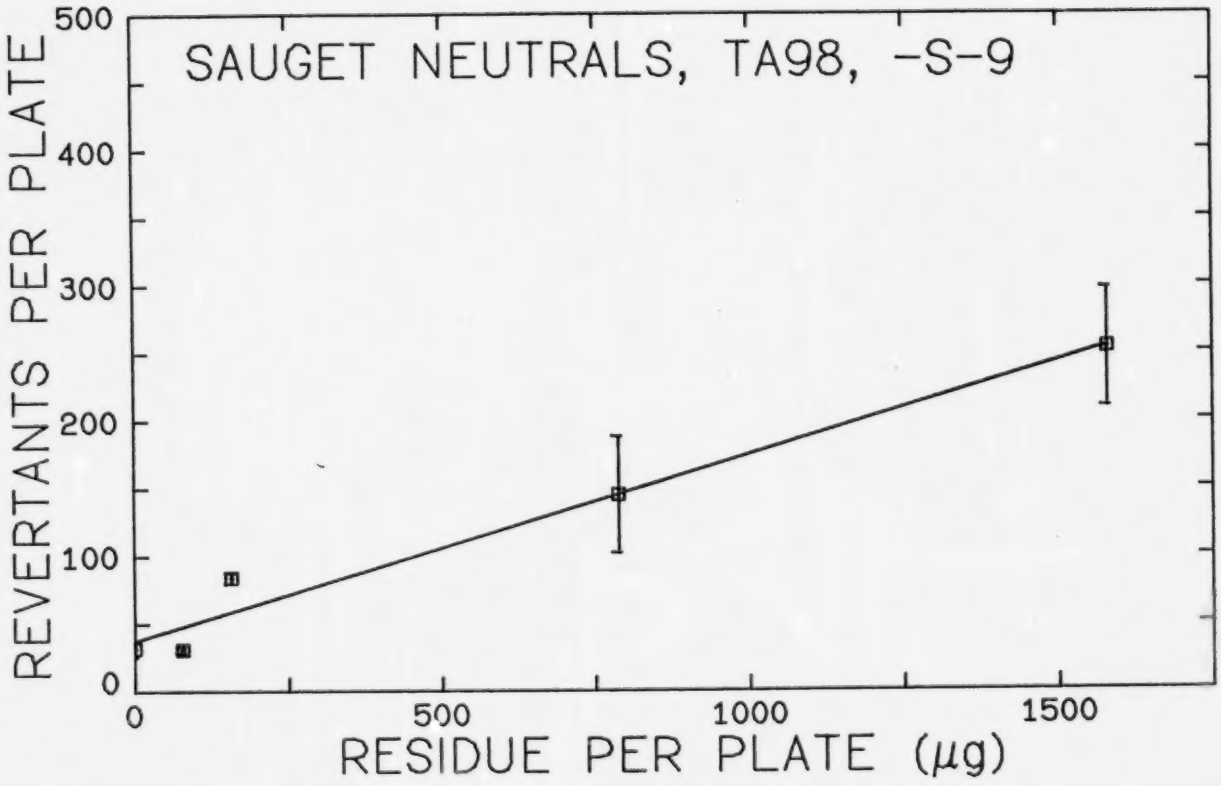

Figure 9. Dose-response curve for Sauget neutral fraction without S-9 activation in strain TA98.

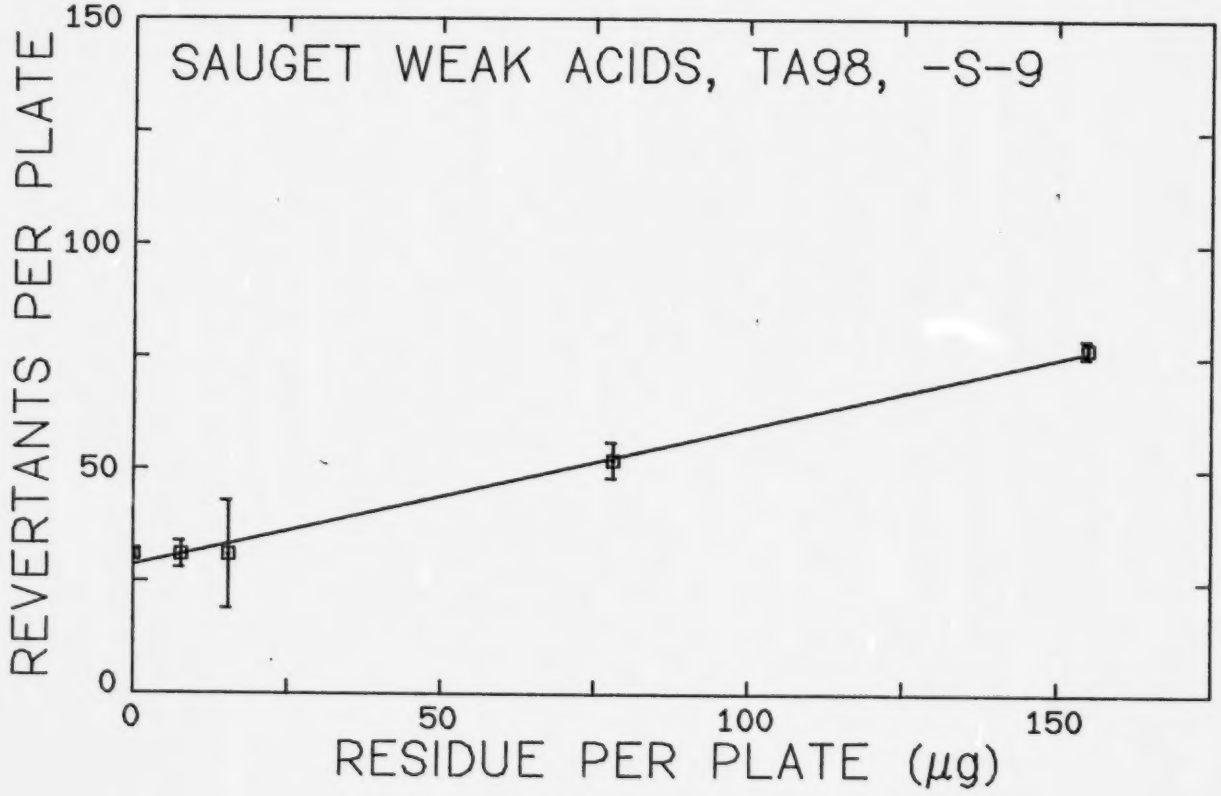

Figure 10. Dose-response curve for Sauget weakly acidic fraction without S-9 activation in strain TA98.

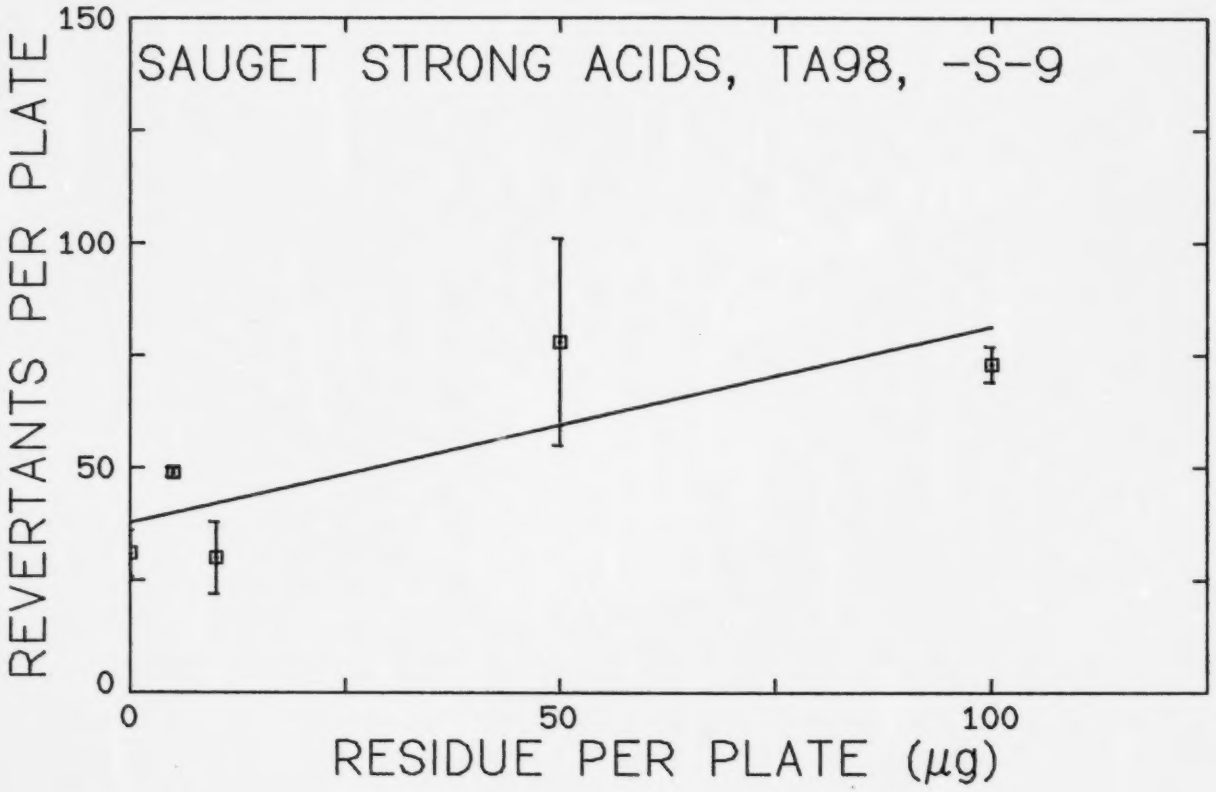

Figure 11. Dose-response curve for Sauget strongly acidic fraction without S-9 activation in strain TA98.

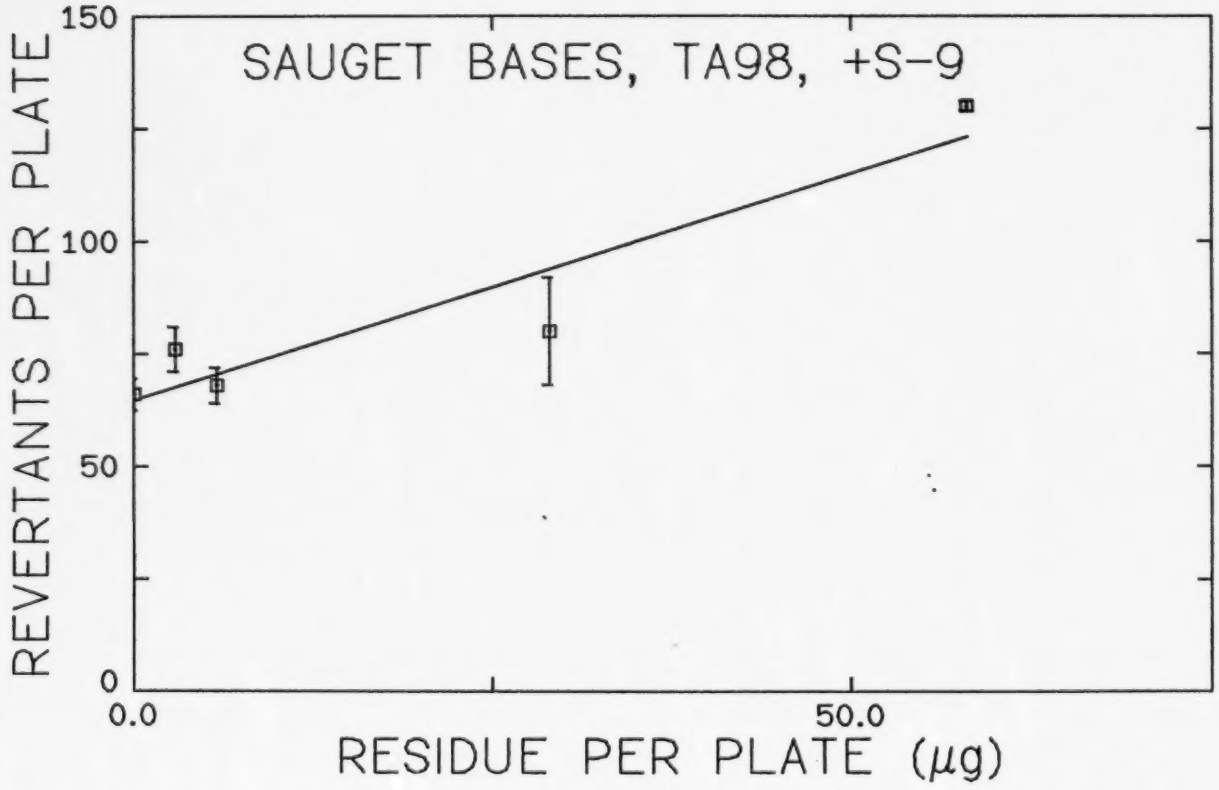

Figure 12. Dose-response curve for Sauget basic fraction with S-9 activation in strain TA98.

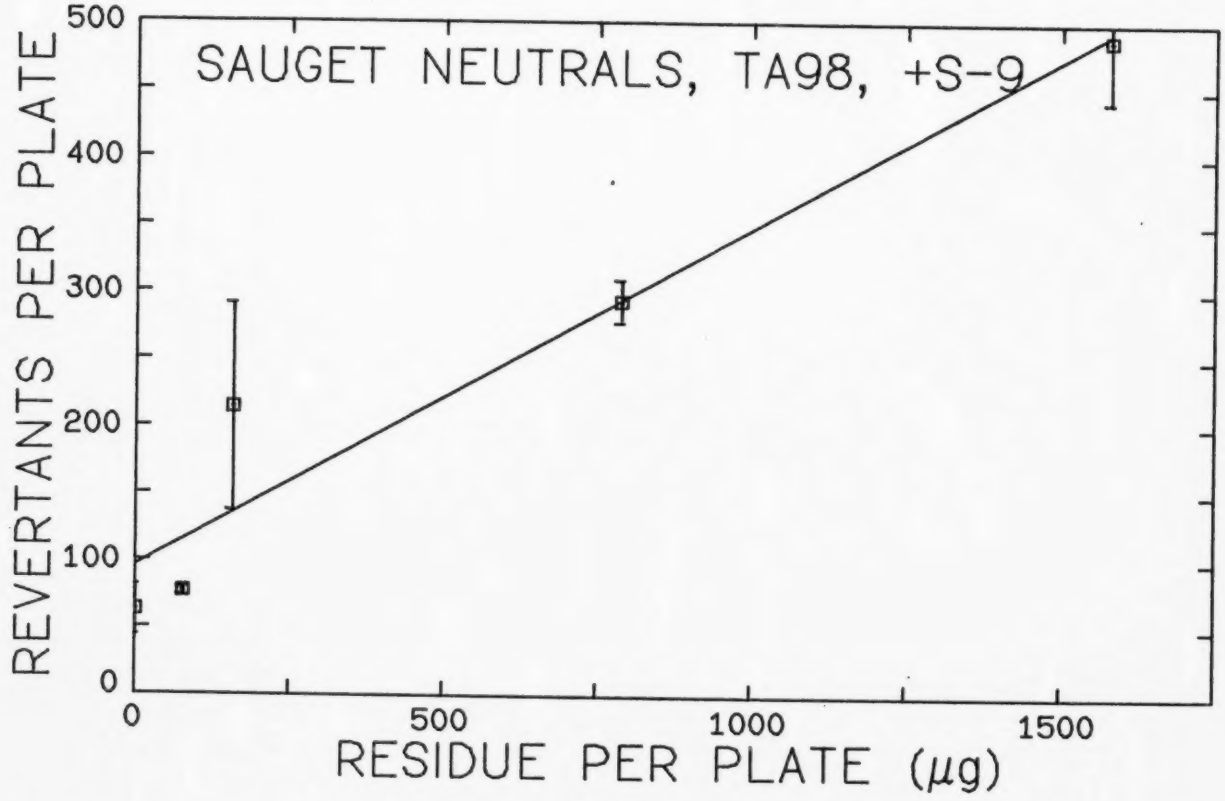

Figure 13. Dose-response curve for Sauget neutral fraction with S-9 activation in strain TA98.

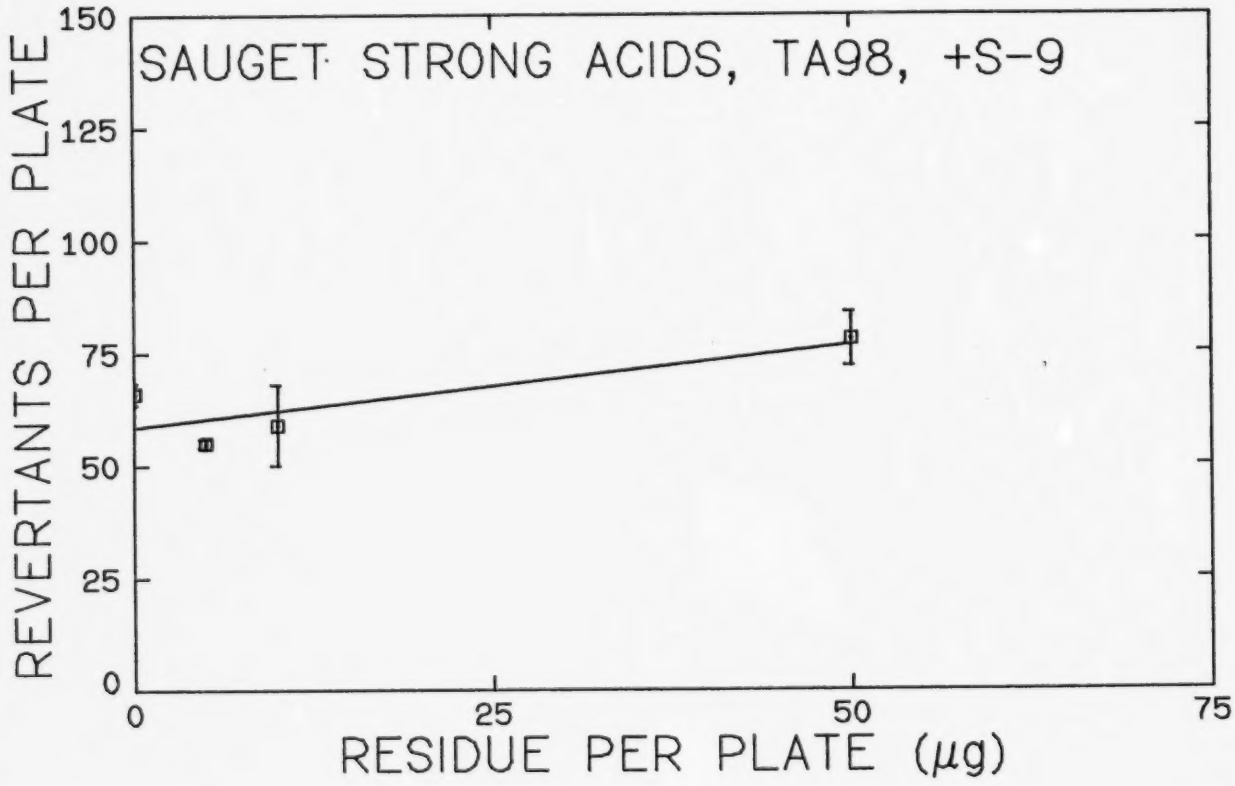

Figure 15. Dose-response curve for Sauget strongly acidic fraction with S-9 activation in strain TA98.

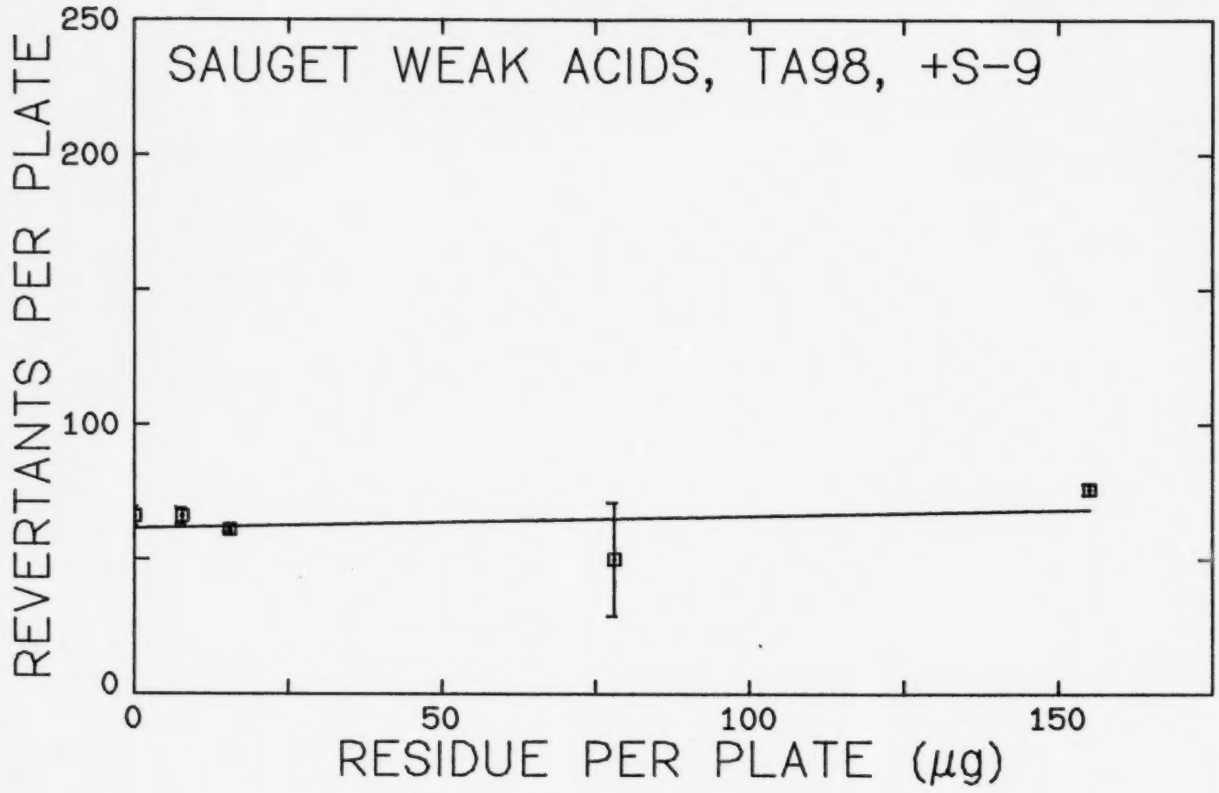

Figure 14. Dose-response curve for Sauget weakly acidic fraction with S-9 activation in strain TA98.

activity with Aroclor/S-9. With Aroclor/S-9, the strongly acidic fraction induced 480 revertants per mg residue whereas without Aroclor/S-9, this fraction induced 380 revertants per mg residue representing a 1.1 fold enhancement.

If the effects of the Sauget fractions to induce reverse mutations were independent, then the sum of the normalized reverse-mutation frequencies should roughly equal the reverse-mutation frequency induced by the crude extract. Table 9 presents the mutagenic activity for each fraction and a normalized value for the reversion-mutation frequencies for each fraction with and without Aroclor/S-9. The sum of the normalized direct acting mutagenicity of these fractions equaled 180 revertants per mg of residue, whereas the crude extract induced 260 revertants per mg of residue. The mutagenic activity of the crude extract is 1.4 fold greater than the sum of the fractions. These data suggests that these fractions act independent of each other. In the presence of Aroclor/S-9, the crude extract induced 3 fold more revertants per mg of residue than could be accounted for by the sum of the reverse-mutation frequencies of the individual extracts. This indicates that these fractions are more mutagenic in the crude extract than as separate entities, suggesting the presence of a synergistic effect(s) among one or more of the fractions.

The neutral fraction of the Champaign, Chicago, and Hinsdale extracts did not demonstrate a mutagenic response in TA98 with or without S-9 mix. Those fractions that did induce a weak or clear mutagenic response were the basic, strongly acidic, weakly acidic fractions. S-9 does not appear to enhance the mutagenic activity of these extracts. The mutagens detected in these sludge samples have ionic character, a more polar solvent system (eg, acetone) has a greater capacity to recovery of mutagens from a wet solid than chloroform:methanol solvent extraction of dry solids.

TABLE 9. MUTAGENICITY OF SAUGET FRACTIONS IN TA98 WITH AND WITHOUT S-9

Fraction	Revertants per mg of Residue -S-9	Revertants per mg of Residue +S-9	% of Crude Extract	Normalized Number of Revertants per mg of Residue -S-9	Normalized Number of Revertants per mg of Residue +S-9
Neutral	129	248	83.4	108	207
Basic	1600	1000	3.1	50	31
Weakly acidic	27.6	--	8.2	2	---
Strong Acidic	380	420	5.3	20	22
Total				180	260
Crude	260	780	100	260	780

SECTION 6

TRADESCANTIA MICRONUCLEUS TEST

BACKGROUND

There are a number of plant assays available to observe genetic damage or change caused by environmental agents. These assays have the advantage of using a higher eukaryotic organism that has a complex genetic structure. Plant systems permit the cost-effective examination of genotoxic effects with relatively high genetic sensitivity. In this and the next section, several different plant systems are described and their application to the study of sludge and sludge-amended soils are presented.

The *Tradescantia paludosa* micronucleus test measures chromosome damage in tetrads following meiosis of the pollen grain mother cells (12,13). A micronucleus may result from the induction of a multipolar nuclear division or by chromosome aberrations that result in acentric fragments. The fragment of broken chromosome can be observed as a separate micronucleus within the tetrad. Figure 16 illustrates both a normal tetrad with each of the four meiotic cells having a single nucleus and a tetrad where two cells contain a micronucleus.

MATERIALS AND METHODS

Inflorescences of *Tradescantia* from clone 03 plants were obtained from T.H. Ma (Western Illinois University, Macomb). The use of a single clone insured that all of the inflorescences were isogenic. The plants were vegetatively propagated and grown under greenhouse conditions. Inflorescences were cut leaving stems approximately 3 to 4 cm long. Typically 8 to 10 inflorescences were used per treatment group. The cuttings were placed in test tubes containing various mixtures of sludge and Hoagland's solution (14). A negative control of Hoagland's solution and a positive control of 50 mM maleic hydrazide in Hoagland's solution were used for each experiment. The inflorescences were treated for 24 hours with the test mixture and then placed into only Hoagland's solution for an additional 24 hours. The solutions were aerated by bubbling water-saturated air through each tube. The temperature was maintained at 24^{o}C using a water bath. After the treatment period, the inflorescences were removed from the treatment tubes and fixed in a solution of ethanol:glacial acetic acid (3:1, v:v) for 48 hours. The samples were then stored in 70 percent ethanol for subsequent analysis.

Figure 16. Photomicrograph of two tetrads of Tradescantia showing a normal tetrad (top) and one with micronuclei (bottom).

The analysis procedure for scoring the micronulei-containing tetrads has been described by Ma (13). The number of multiple micronuclei per tetrad was also recorded. The frequency of micronuclei induction was determined by dividing the total number of micronuclei observed by the number of tetrads analyzed. This ratio was multiplied by 100 to yield the number of micronuclei per 100 tetrads. As in most mutagenicity assays, a twofold increase in the rate of induction of micronuclei is considered significant.

The sludge samples and sludge fractions assayed with the Tradescantia micronucleus test are presented in Table 10.

Chicago Sludge

The results of the micronucleus test on whole Chicago sludge and various dilutions are presented in Table 11. The data clearly demonstrate a dose dependent increase in the frequency of micronuclei with increased sludge concentration. As illustrated in Figure 17, dilutions above 1/4 induced a significant increase in the frequency of micronuclei. Whole sludge, the 1/2 dilution and the positive control induced an increased frequency of tetrads containing multiple micronuclei. Thus, whole Chicago sewage sludge and dilutions above 1/4 can induce cytogenetic damage in meiotic cells including multiple events in single tetrads.

An aqueous phase of Chicago sludge was prepared by removing the water from 10 liters of the sludge aqueous fraction by vacuum distillation and lyophilization. The resulting dry material was redissolved in 200 ml of distilled water. This solution was a 50x concentration of the original sludge aqueous fraction. Sterilized and nonsterilized Chicago sludge aqueous phase as well as 5x, 12.5x and 25x concentrations were analyzed with Tradescantia. The data are presented in Table 12. A dose dependent increase in the frequency of micronuclei was observed with the increase in the concentration factor of the Chicago sludge aqueous fraction. It is interesting to note that the aqueous fraction is much less mutagenic than whole sludge. An unconcentrated or a 5x concentrated aqueous phase sample did not induce a significant increase in the frequency of micronuclei.

A series of tests were conducted on the acetone fraction of Chicago sewage sludge. The fraction was prepared by vacuum distilling an acetone extract of solid sludge material. The residue was redissolved in 0.5 ml DMSO. The DMSO solution was mixed with 9.5 ml of Hoagland's solution and assayed. No induction of micronuclei was detected with the acetone fraction (Table 12).

The Tradescantia tests with Chicago sludge indicate that agents are present that can inflict cytogenetic damage in meiotic cells of a higher eukaryote. It is interesting to note that the acetone fraction did not

TABLE 10. MUNICIPAL SEWAGE SLUDGE SAMPLES OR SLUDGE FRACTIONS ASSAYED WITH THE TRADESCANTIA MICRONUCLEUS TEST

Sample	Concentration	Response
Negative Control	Hoagland's Solution	-
Positive Control	50 mm Maleic Hydrazide	+
Whole Chicago Sludge	1/16 Dilution	-
	1/8 Dilution	-
	1/4 Dilution	-
	1/2 Dilution	+
	1/1 Dilution	+
Chicago Sludge Aqueous Phase	Nonsterilized	-
	Sterilized	-
	5x Concentration	-
	12.5x Concentration	+
	25x Concentration	+
Chicago Sludge Acetone Fraction		-
Champaign Sludge	Whole Sludge	-
	1/2 Dilution	-
Concentrated Sludge	1.0x	-
	2.0x	-
	4.0x	-
Kankakee Sludge	Whole Sludge	-
Concentrated Sludge	6.7x	-
	13.3x	-
	20.0x	+
Hinsdale Sludge	Whole Sludge	-
Concentrated Sludge	6.7x	-
	13.3x	-
	20.0x	-

TABLE 11. INDUCTION OF MICRONUCLEI IN TRADESCANTIA BY CHICAGO MUNICIPAL SEWAGE SLUDGE

Treatment	Number of Tetrads Analyzed	Total Number of Micronuclei	Micronuclei per 100 Tetrads	Frequency of Multiple Micronuclei ($x10^3$)
EXPERIMENT 1				
Control	9980	337	3.4	2.7
1/16 Dilution	4654	160	3.7	3.2
1/8 Dilution	4958	185	3.7	1.8
1/4 Dilution	6471	234	3.6	2.2
1/2 Dilution	5691	273	4.8	4.7
EXPERIMENT 2				
Control	6673	236	3.5	2.8
50 mM Maleic Hydrazide	3176	358	11.3	6.0
Whole Sludge	2355	199	8.5	4.2
EXPERIMENT 3				
Control	8056	294	3.6	3.6
50 mM Maleic Hydrazide	1906	226	11.8	4.7
1/4 Dilution	5655	206	3.6	1.4
1/2 Dilution	7011	405	5.8	4.0
Whole Sludge	10627	889	8.4	7.8
EXPERIMENT 4				
Control	10452	336	3.2	0.9
50 mM Maleic Hydrazide	4517	425	9.4	9.1
1/4 Dilution	8656	301	3.5	2.5
1/2 Dilution	5955	290	4.9	2.9
Whole Sludge	2596	255	9.8	10.0

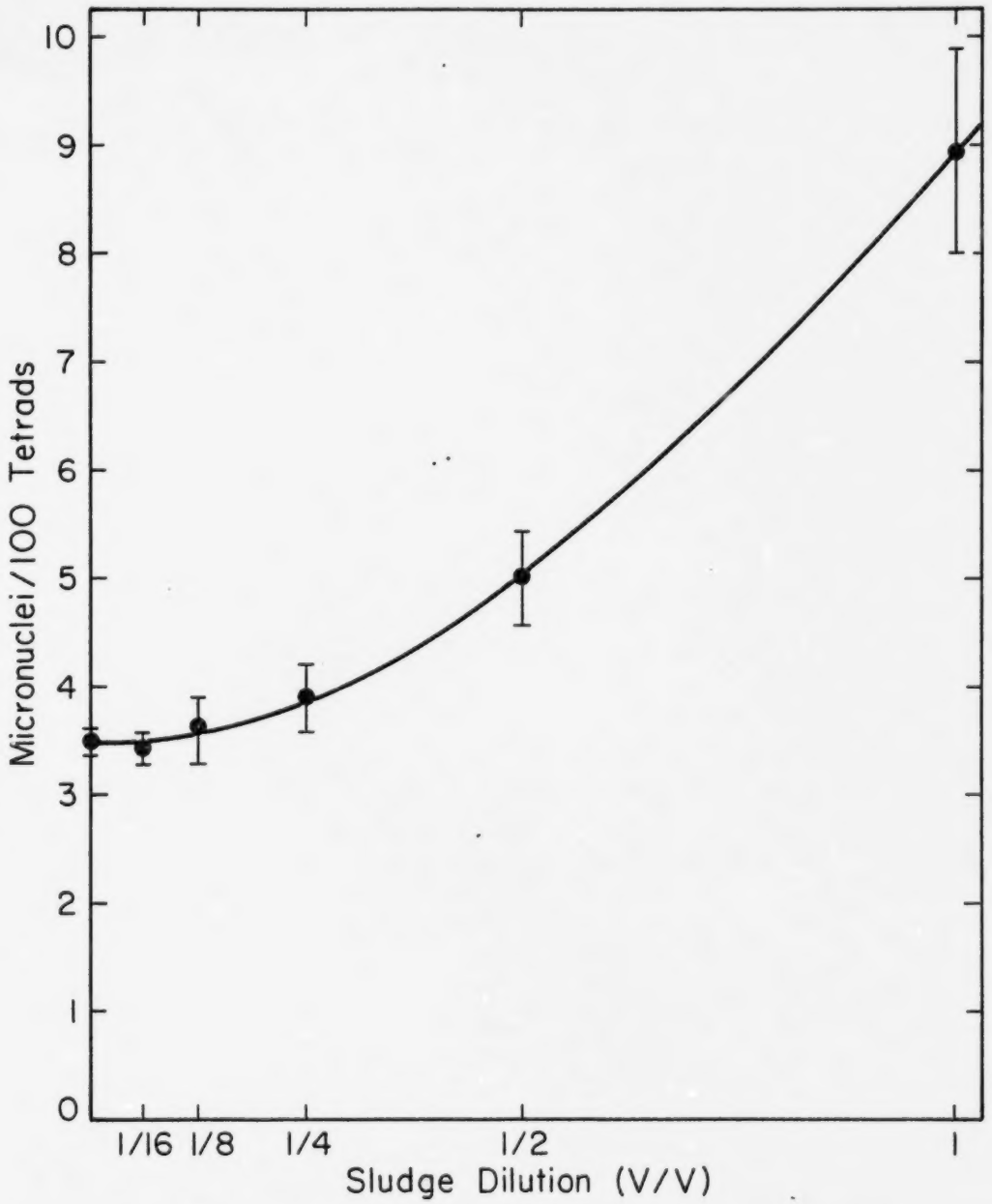

Figure 17. Induction of micronuclei in tetrads of Tradescantia as a function of Chicago sludge concentration. The data from the four experiments have been averaged and the error bars represent one standard error of the mean.

TABLE 12. RESULTS OF THE CHICAGO SLUDGE AQUEOUS FRACTIONS AND ACETONE EXTRACTS WITH THE TRADESCANTIA MICRONUCLEUS TEST

Treatment	No. Tetrads	No. MCN	MCN/100 Tetrads
Control	10,750	340	3.2 ± 0.50
Aqueous Phase (Nonsterilized)	23,514	813	3.5 ± 0.30
Aqueous Phase (Sterilized)	27,347	1,014	3.7 ± 0.50
Aqueous Phase (25x)	7,531	664	8.8 ± 2.40
Acetone Fraction	13,013	705	5.4 ± 1.10
Control	35,440	1,264	3.6 ± 0.51
Aqueous Phase (5x)	15,300	760	5.0 ± 0.77
Aqueous Phase (12.5x)	12,298	952	7.7 ± 1.50
Aqueous Phase (25x)	8,175	866	10.6 ± 0.84
Acetone Fraction	23,050	927	4.0 ± 0.43

induce micronuclei. At the concentration tested, it is apparent that some agents in the sludge that induce point mutations in bacteria are unable to induce chromosomal damage in meiotic cells.

Champaign Sludge

Two samples of whole sludge were assayed for their ability to induce micronuclei. Neither sample induced a significant increase in cytogenetic damage (Table 13).

Whole sludge was concentrated by lyophilization. The solid sludge was then mixed with Hoagland's solution at rates of 0.3 g:4.7 ml and 0.6 g:4.7 ml which corresponded to approximately 2x and 4x concentrations, respectively. The results of these experiments are presented in Table 14 and none of the Champaign sludge samples induced a significant increase in the frequency of micronuclei.

Hinsdale Sludge

Whole sludge samples were assayed and a negative response was observed (Table 13). The Hinsdale sludge was concentrated by lyophilization. The sludge solids were mixed with Hoagland's solution at rates of 1 g:5 ml, 2 g:5 ml and 3 g:5 ml which corresponded to 6.7x, 13.3x and 20.0x concentrations, respectively. The data for the concentrated Hinsdale sludge are presented in Table 14. The concentrated Hinsdale sludge samples did not significantly increase the induction of micronuclei. These data agree with the results obtained with the unconcentrated samples of Hinsdale sludge.

Kankakee Sludge

Kankakee whole sludge samples were assayed and found to be negative (Table 13). Whole sludge was concentrated by lyophilization and 6.7x, 13.3x and 20.0x concentrations in Hoagland's solution were prepared and assayed. The results are presented in Table 14 and only the 20x sludge concentration induced a positive response. Thus, it appears that a low concentration of mutagen(s) is present in Kankakee sludge that can induce chromosome damage in meiotic cells of Tradescantia.

TABLE 13. RESULTS OF DIFFERENT WHOLE SLUDGE SAMPLES FROM FOUR CITIES WITH THE TRADESCANTIA MICRONUCLEUS TEST

Sample	No. Tetrads	No. MCN	MCN/100 TETRADS
Control	6,174	180	2.92 ± 0.55
+ Control (50 mM MH)	8,887	972	10.94 ± 1.42
Chicago Sludge	8,071	515	6.38 ± 1.61
Kankakee Sludge	7,048	347	4.92 ± 0.80
Hinsdale Sludge	6,333	314	4.48 ± 0.74
Champaign Sludge (Sample 1)	8,225	342	4.16 ± 0.57
Champaign Sludge (Sample 2)	6,460	282	4.37 ± 0.57

TABLE 14. RESULTS OF CONCENTRATED SLUDGE SAMPLES FROM SEVERAL CITIES WITH THE TRADESCANTIA MICRONUCLEUS TEST

Sample	No. Tetrads	No. MCN	MCN/100 Tetrads
Control	5,297	200	3.8 ± 0.60
+ Control (50 mM MH)	7,989	445	5.6 ± 1.35
Kankakee Sludge (6.7x)	5,486	348	6.3 ± 0.88
Kankakee Sludge (13.3x)	6,408	404	6.3 ± 0.76
Kankakee Sludge (20.0x)	3,027	298	9.8 ± 2.71
Control	10,449	539	5.2 ± 0.63
+ Control (50 mM MH)	15,032	1,263	8.4 ± 1.57
Hinsdale Sludge (6.7x)	10,030	610	6.1 ± 0.86
Hinsdale Sludge (13.3x)	6,271	319	5.1 ± 1.00
Hinsdale Sludge (20.0x)	7,202	447	6.2 ± 1.10
Champaign Sludge	14,135	1,059	6.6 ± 0.78
Champaign Sludge (2x)	10,263	460	4.5 ± 0.57
Champaign Sludge (4x)	10,051	533	5.3 ± 1.03

SECTION 7

MAIZE *wx* LOCUS ASSAY

BACKGROUND

The waxy (*wx*) locus is located on chromosome 9 of *Zea mays* at map position 9-59 (15). The *wx* locus controls the synthesis of the starch amylose. Pollen grains (microgametophytes) are functional haploids and they express their genetic constitution rather than that of their parental sporophyte. A pollen grain that carries a dominant allele (*Wx*) can synthesize amylose as part of its starch component. Amylose when reacted with iodine turns a dark blue-black color (16) and *Wx* grains will therefore stain blue-black. A pollen grain that carries the recessive allele (*wx*) cannot synthesize amylose and will appear tan when reacted with iodine. Thus, the color of the pollen grain after staining with iodine gives a clear indication of its genotype. Thus, genetic reversion of *wx* to *Wx* can be quantitatively determined by counting the number of black stained pollen grains within the field of tan stained grains. In the forward mutation assay, *Wx* to *wx*, tan stained forward mutant grains are counted in a field of black stained pollen grains. In each case the frequency of mutant pollen grains is determined by counting individual mutant grains and estimating the number of viable pollen grains by counting the number present in 20 randomly selected 1 mm^2 areas and multiplying by the slide area. The use of the maize *wx* locus assay for *in situ* and laboratory studies of mutagenesis have been reviewed by Plewa (17) and Plewa and Wagner (18).

It can be determined if there are statistically significant increases in the frequencies of mutant pollen grains between the control and treatment groups by using the $\emptyset$ statistic described by Katz (19). Katz has adapted the test specifically for the pollen grain assay (20). The test statistic, $\emptyset$, is defined by

$$\emptyset = \frac{n(m - 0.5) - N(m + 0.5)}{[nN(m + M)]^{1/2}}$$

where m is the observed number of mutant pollen grains in the control group, M is the observed number of mutant grains in a treatment group, n is the estimated number of pollen grains analyzed in the control group, and N is the estimated number of pollen grains in the treatment group. A one-tailed test for an 0.05 level of significance would be obtained for $\emptyset > 1.64$.

REVERSE MUTATION TEST IN IN SITU STUDIES

MATERIALS AND METHODS

An *in situ* study of the possible mutagenic effects of municipal sewage sludge was conducted between May and August 1979. Test plots were constructed on the NW900 plots at the University of Illinois Agronomy Research Center near Elwood, Illinois. A history of these plots covering the period 1968 to 1979 is presented in Table 15. If *in situ* studies are to be valid, it is important to know the past history of such plots since prior application of mutagenic substances could seriously interfere with the planned studies. It is particularly important to know that the sample and control areas have the same history so that the controls properly reflect the base conditions of the study.

Z. mays kernels of inbred M14 homozygous for either the *wx-C* or the *wx-90* allele obtained from the maize nursery of M. Plewa were siblings and inbred for over 12 generations to insure a high level of isogeneity. The heteroalleles *wx-C* and *wx-90* are different mutons within the *wx* cistron. They occupy separate and different mutational sites within the gene. It has been suggested that *wx-C* is a base pair substitution (21). The molecular nature of the *wx-90* heteroallele is unknown. One row of each allele type was planted in a control plot and in each of three treatment plots. The maximum treatment group received 17.8 cm of liquid sludge equivalent to 21.0 t/ha of dry material over the growing season. The other treatment groups received one-half and one-quarter of this amount, respectively. The control plot received adequate nutrients by the application of chemical fertilizer. No insecticides or herbicides were applied to these plots.

The plants grew until early anthesis when each tassel was individually labeled, harvested, and stored in 70 percent ethanol for subsequent analysis. Pollen grains were removed from the tassels and analyzed as described by Plewa (17). The frequency of revertant pollen grains was determined by dividing the total number of revertant grains by the total number of viable pollen grains. The percentage of clear, collapsed, aborted pollen grains was also determined by estimating the number of aborted pollen grains and dividing by the sum of the viable plus aborted grains.

RESULTS

The data for the induction of revertant pollen grains at the *wx-C* and *wx-90* alleles from *Z. mays* grown in the sludge test plots are presented in Table 16. For the *wx-C* allele, over 5.1 million pollen grains were analyzed. The frequencies of reverse mutation decreased with the increased amount of sewage sludge added to the soil. A direct relationship was observed between the amount of applied sludge and the frequency of pollen abortions within the range of 19.4 percent for the control to 49.9 percent in the highest treatment group. Part of the

TABLE 15. HISTORY OF THE NW 900 PLOTS AT THE UNIVERSITY OF ILLINOIS AGRONOMY RESEARCH CENTER

Year	Treatment
1968	Plots received 61.06 t/ha sludge solids.
1969	Plots plated to kenaf, received 41.48 t/ha sludge solids.
1970	1.9 l. Eptam 6E was applied and disced into the soil and alfalfa was seeded. 32 grams dalapon (Dowpon) in 5.7 l. water was applied to the sludge plots. Plots received 28.38 t/ha sludge solids.
1971	No sludge applied. Plots in alfalfa and Dowpon sprayed on grassy areas for grass control.
1972	Plots in alfalfa. Plots received 12.16 t/ha sludge solids.
1973	Plots in alfalfa. Plots received 20.77 t/ha sludge solids.
1974	No crop. Plots received 46.21 t/ha sludge solids.
1975	No sludge applied. Plots in alfalfa.
1976	No sludge applied. Plots in spinach.
1977	No sludge applied. Plots in spinach.
1978	No sludge applied. Plots in spinach.
1979	Plots Numbers 5, 13, 14, and 15 used in the maize wx locus assay.

TABLE 16. SUMMARY OF DATA FOR wx REVERSION IN THE IN SITU TESTS

Experiment Number	No. Mutant Pollen Grains	No. Viable Pollen Grains	% Abortion	Frequency of Mutant Pollen Grains	Statistic	
wx-C inbred M14						
4336-C	37	1,267,963	19.4	2.9×10^{-5}		
4338	17	1,052,855	36.5	1.6×10^{-5}	-2.18	$p<0.01$
4340	5	1,429,642	46.5	3.5×10^{-6}	-5.49	$p<0.001$
4342	6	1,299,568	49.9	4.6×10^{-6}	-4.96	$p<0.001$
wx-90 inbred M14						
4335-C	2	1,266,242	27.9	1.6×10^{-6}		
4337	2	1,268,930	26.6	1.6×10^{-6}	-0.50	$p>0.05$
4339	4	1,256,245	29.9	3.2×10^{-6}	0.42	$p>0.05$
4341	22	1,212,922	28.2	1.8×10^{-5}	3.98	$p<0.001$

reason for the decreasing rate of observed mutations may be the apparent toxicity of the sludge to this maize strain. If the pollen grains are killed, they cannot express mutation.

The relationship between the abortion frequency in plants homozygous for wx-C and concentration of amended sludge indicated a increasing rate of gametophytic death. Thus, for this allele some agent in the sludge was toxic to the developing pollen grains. No increased frequency of revertants in the wx-C allele was seen and may be due in part to the observed toxicity.

A different situation was obtained for the wx-90 reversion tests. For wx-90 there was an increased frequency of revertants with increased sludge amendment for the half-maximum and maximum treatment plots. In the control plots, the frequency of revertants was 1.6×10^{-6} : plants grown in the half-maximum sludge plots exhibited a reversion frequency that was twice that of the controls. There was a ten-fold increase in the frequency of revertants for the highest treatment group. The percentage of pollen abortions was essentially constant among the control and treatment groups. The sludge was apparently not toxic to the developing pollen grains in this inbred line. These data indicate that these two alleles express different sensitivities to constituents in the sludge. A difference in the specific types of lesions may account for the differences in response to exposure to sludge.

FORWARD MUTATION TEST IN EARLY-EARLY SYNTHETIC

MATERIALS AND METHODS

The forward mutation test was conducted to detect a broader range of mutational events and to allow the administration of higher concentrations of sludge than were possible in the *in situ* assays. Inbred Early-Early Synthetic was chosen because its convenient growth characteristics permit its use in plant growth chambers where well-controlled conditions can be assured. The genetic endpoint of this assay is the forward mutation at the *wx* locus in pollen grains. The plants used in this test were homozygous for the dominant *Wx* allele. Mutation is to the recessive *wx* allele, and the presence of tan grains after treatment with iodine is the visual endpoint.

Inbred Early-Early Synthetic *Z. mays* kernels were obtained from sibling crosses and are homozygous for the dominant *Wx* allele. Five plants were grown as a control group in individual 10-cm-diameter plastic pots filled with a standard soil mixture and watered with deionized water. Standard potting soil consists of four parts loam, two parts peat moss and one part sand. Once a week 50 ml of a 20:20:20 nitrogen:phosphorus:potash fertilizer solution were added to each pot. Three treatment groups of five plants each were prepared with differing amounts of sludge added to the initial soil mixture. For the highest treatment group, one part sludge was added to two parts of standard soil. These plants were watered with a 1/3 dilution of sludge in deionized water. For the next treatment group, one part sludge was added to 5 parts of soil. The plants were watered with a 1/6 dilution of sludge. In the lowest treatment group, 11 parts of soil were combined with one part of sludge and these plants were watered with a 1/12 sludge/deionized water solution. The treatment groups were grown in a separate chamber from the control plants. The chambers were set for a 17-hour photoperiod of 300 μEinsteins/m^2/sec with day and night temperatures of 25°C and 20°C, respectively. The plants in the treatment groups were watered with the appropriately diluted sludge until tassel emergence and then watered with deionized water only. At early anthesis the tassels were harvested, labeled, and stored in 70 percent ethanol for subsequent analysis. The pollen grains were then analyzed in the same manner as described above except that forward mutants (*Wx* to *wx*: black to tan pollen grains) were scored.

RESULTS

The results of the induction of forward mutation at the *wx* locus in Early-Early Synthetic maize for the Chicago sludge sample are presented in Table 17. The data clearly show that the administration of Chicago sludge increased the frequency of mutation by two orders of magnitude. The control frequency of mutant pollen grains of 4.3×10^{-5} is consistent with spontaneous frequencies observed in other studies with this inbred (18). The frequencies of mutant pollen grains decreased

TABLE 17. FORWARD MUTATION AT THE MAIZE *wx* LOCUS INDUCED BY CHICAGO SEWAGE SLUDGE

Treatment	Number of Mutants	Number of Pollen Grains Analyzed	Frequency Mutant Pollen Grains	% Pollen Abortion	Statistic
Control	34	788,000	4.3×10^{-5}	13.3	
Low (1/12) Concentration	7585	825,000	9.2×10^{-3}	17.1	$p<0.001$
Medium (1/6) Concentration	5650	898,000	6.4×10^{-3}	20.4	$p<0.001$
High (1/3) Concentration	1776	836,000	2.1×10^{-3}	19.9	$p<0.001$

with increasing amounts of added sludge indicating that even the lowest concentration of applied Chicago sludge is high enough to reach the toxic region of the mutational dose-response curve. The percentage of pollen abortion is higher at the higher sludge concentrations than the control group. The control frequency is 13.3 percent while it is 19.9 percent in the highest treatment group. There is, however, no dose-dependence to the abortion data. There is a clear indication that Chicago sludge induced forward mutation in the pollen grains with a substantial increase in frequency over the control group value. The increased abortion rate indicated the presence of toxic as well as mutagenic agents.

Within the statistical limits of the experiment, the Champaign sludge sample did not induce an increased frequency of forward mutations in this assay. These results are in agreement with the other assays that show that Champaign sludge does not contain mutagens.

SECTION 8

SISTER-CHROMATID EXCHANGE ASSAY

BACKGROUND

During the late 1950's, Taylor and his coworkers unequivocally demonstrated the phenomenon of sister chromatid exchanges in mitotic chromosomes of the angiosperm Vicia faba (22). A chromatid is one of two daughter strands of a duplicated, or G2 chromosome which are joined by a single centromere. A sister chromatid exchange results when there is a physical and reciprocal exchange of chromatin between the two daughter chromatids of a single chromosome. Most of the early molecular cytogenetic studies on sister chromatid exchanges were conducted by radioactively labeling the chromosomal DNA with tritium for two rounds of chromosomal replication and analyzing metaphase chromosomes by autoradiography. In such chromosomes, one chromatid could be distinguished from the other. Perry and Wolff (23) developed the fluorescence plus Giemsa (FPG) technique for sister chromatid exchange analysis. This technique has nearly replaced the use of tritium as a label for DNA. The halogenated DNA base analog, 5-bromo-2'-deoxyuridine (BUdR) is used to label DNA. The BUdR is substituted for some of the thymidine in the DNA of the replicating chromosomes. The interaction of BUdR and a fluorochrome stain causes a differential coloring between the bifilarly substituted chromatid and the unifilarly substituted chromatid. The ability to resolve the bifilarly and unifilarly substituted chromatids of a single chromosome is due to the fact that DNA replicates semiconservatively. It has been demonstrated that chemicals that induce mutations and/or cancer also increase the frequency of sister chromatid exchanges in mammalian or plant cells (24). Studies indicate that sister chromatid exchange frequencies are more sensitive indices of genetic damage than frequencies of chromosomal aberrations (25).

MATERIALS AND METHODS

Blood was drawn by a trained phlebotomist from human volunteers with Vacutainer tubes containing 2% heparin (1,000 units/ml). The use of human subjects in this project was approved by the institutional review board of the University of Illinois. Blood (in 5 ml amounts) was transferred to sterile, capped test tubes and centrifuged for 5 min at 1,000 rpm. The buffy coat (the layer which contains the lymphocytes)

was removed from each tube and mixed with the conditioned tissue culture medium. The tissue culture medium consisted of 70% RPMI medium 1640 and 30% calf serum and was conditioned by placing in a 37°C incubator for 1 h prior to its use. The amount of tissue culture medium prepared was determined by the experimental design and the amount of blood drawn. Two disposable petri plates (diameter of 6.3 cm) were prepared for each 5 ml portion of blood drawn and each plate contained 5 ml of the medium plus the buffy coat. One hundred and fifty µl of phytohaemagglutinin (PHA) was added to each plate to induce the agglutination of erythrocytes and to stimulate mitosis in the lymphocytes. Each plate also contained 200 µM 5-bromo-2'-deoxyuridine (BRdU). all of the above procedures were conducted under yellow lamps to prevent the degradation of the BRdU. The cells were incubated for 73 h at 37°C under 5% CO_2. After the cells proceed through two rounds of DNA replication, a chromatid of each chromosome has BRdU substituted for thymidine in both strands of its DNA while the other chromatid has only one strand of its DNA substituted with BRdU. The cells must be in metaphase to visualize the chromosomes. To insure the proper sequencing of events, 0.2 µg of the mitotic inhibitor colchicine was added to each plate after 70 h of incubation.

To evaluate the genotoxicity of a substance the cells were exposed to the test agent for 1 or 2 h after 48 h in tissue culture. At that time, the contents of each petri plate were transferred into sterile, capped conical centrifuge tubes. The cells were centrifuged for 5 min at 1,000 rpm. The supernatant fluid was decanted and the cells were agitated with 5 ml of sterile warm 10% Hank's balanced salt solution. This procedure prevented any reaction of the test agent with proteins in the tissue culture medium. The test agent was added to the tubes and agitated. The cells were exposed to the test agent with or without a mammalian microsomal activation mixture (S-9). When S-9 activation was included, one ml of S-9 mixture was added with the test agent. The S-9 was prepared with the hepatic supernatant fluid (9,000 xg) obtained from rats induced with Aroclor 1254. Each ml of S-9 mixture contained 168 µmoles of sodium phosphate, 50 µmoles of NADP, 40 µmoles of sodium glucose-6-phosphate, 33 µmoles of potassium chloride, 8 µmoles of magnesium chloride, and 3 mg protein of hepatic S-9 fraction (700 µl of a 43 mg protein/ml). After addition of the test agent, cells were transferred into petri plates. After 1 or 2 h incubation, the cells were transferred to test tubes and centrifuged at 1,000 rpm for 5 min. The supernatent fluid was removed and the cells were washed twice with 5 ml of a medium of warm 85% RPMI and 15% calf serium. The cells were resuspended in a warm medium of 30% calf serium with 70% RPMI and poured into petri plates with 200 µM BRdU. The cells were incubated for an additional 25 or 27 h.

After a total of 73 or 76 h, the lymphocytes were harvested. the contents of each plate of medium and cells were transferred to separate centrifuge tubes and centrifuged for 5 min at 1,000 rpm. The medium was decanted and the cells were washed with 5 ml of a 10% Hank's balanced salt solution and recentrifuged. The cells were resuspended in 7 ml of

a 0.075 M KCl solution for 15 min, causing the erythrocytes to burst and the lymphocytes to swell. The cells were centrifuged and the pellet agitated with 5 ml of a freshly prepared fixative of methanol:acetic acid (3:1 v/v). The cells were centrifuged and the supernatant fluid decanted to leave approximately 0.25 ml. A small, white pellet of cells remained in the bottom of the centrifuge tube. The pellet was disrupted to prepare a suspension of cells and three to four drops of the cell suspension were dropped from a height of 750 cm on an ice cold microscope slide. The slides were placed on a slide warmer at 60°C for 1-2 min and allowed to air dry under fluorescent lamps for 2-3 days.

The differential staining of the chromosomes was induced by the fluorescence plus Giemsa technique (FPG). Three to four drops of the fluorochrome dye (Hoechst 33258: 0.50 ug/ml deionized, distilled water) were placed on each slide. A cover slip was placed on each slide and kept in room light for 20 min. The cover slips were rinsed off with deionized water and the slides were air dried. Several drops of a citric phosphate buffer (pH 7) were placed on each slide and cover slips were replaced. The slides were exposed to ultraviolet light (GE F15T8-B1) for 30 min. The cover slips were removed and the slides were placed in 2x ssc (saline sodium citrate buffer, 0.3 M NaCl and 0.03 M sodium citrate) at 62° C for 90 min. The slides were immediately stained with gurr Giemsa (3% in citric phosphate buffer) for 8 min. The slides were dipped in citric phosphate buffer to rinse off excess stain, dried and permanently mounted.

After the slide mounting cement hardened, each slide was examined under a Zeiss Universal microscope with brightfield illumination (543 nm) at a magnification of 1,260 x. Only well-differentiated metaphase chromosomes were scored for sister chromatid exchanges (SCE). Cell preparations with a least 41 chromosomes were used in the analysis of SCEs. Cells with at least 41 chromosomes may be used for the scoring of SCE'S because there is no significant difference in the mean SCE frequency among cells with 41 to 46 chromosomes (26). For each properly stained spread, the chromosomes were counted and SCEs were scored when a reciprocal exchange of stain was observed. A reciprocal exchange of stain is defined as a darkly stained region on one chromatid accompanied by a lightly stained region in the identical, reciprocal region of the sister chromatid.

RESULTS

The Chicago sludge extracts have been examined with sister chromatid exchange analysis. In one series of experiments, human cells were exposed to acetone extracts of Chicago sludge E1a, E1b, and E1c. These extracts were very toxic and substantially altered the growth cycle of these cells. DMSO controls were run since the sludge extracts are suspended in DMSO. A 100 µl sample of DMSO induced a frequency of 0.17 SCE/chromosome. Ethylmethane sulfonate (EMS) was used as the positive

control for all these experiments. The historical control frequency for 5 mM EMS/plate is 0.33 SCE/chromosome. The results are presented in Table 18. These results indicate that the sludge extracts tested do not induce SCE's as none of the values are significantly above the normal control frequency. The results also show that E1a is toxic to human lymphocytes at high concentrations.

A second experimental series was conducted in which the exposure time was lengthened to 2 h and the total incubation time increased to 76 h. This increased incubation time permitted the treated cells to complete their second cell division. Fraction E1c was tested at three concentrations: 0.1x, 1x, and 15x. A 100 µl sample of each concentration of this extract (E1c) as well as positive and negative controls were assayed. The negative control had a frequency of 0.21 $\pm$ 0.02 SCE/chromosome while the 5 mM EMS positive control induced a frequency of 0.48 $\pm$ SCE/chromosome. The number of chromosomes scored for the negative and positive controls were 1538 and 1469, respectively. The unweighted mean SCE frequency for the E1c 0.1x, 1x, and 15x concentrations were 0.21 $\pm$ 0.04, 0.26 $\pm$ 0.03, and 0.32 $\pm$ 0.02 SCE/chromosome, respectively (Table 18). The highest E1c concentration induced a statistically significant ($p<0.01$) increase in the SCE frequency as compared to the negative control. There also appears to be a dose-dependent increase in the SCE frequency. Thus, in fraction E1c, there is a genotoxin(s) that can induce a significant increase in sister chromatid exchanges in human lymphocytes. The complete set of experiments to assess the mutagenicity of the sludge extracts are in progress and the results will be submitted for publication when they are aavailable.

TABLE 18. GENOTOXICITY OF MUNICIPAL SEWAGE SLUDGE FRACTIONS IN HUMAN LYMPHOCYTES

Sample	Volume culture, (µl)	Concentration Factor	SCE/Chromosome (72 h culture, 1 h exposure)	SCE/Chromosome (76 h culture, 1 h exposure)
E1a	100	0.0	0.17	0.20
		0.1	0.21	0.23
		1.0	cell death	0.23
		10.	cell death	0.18
		15.	cell death	0.26
E1b	100	0.0	0.17	0.20
		0.1	0.19	0.22
		1.0	0.16	0.24
		10.	0.20	0.27
		15.	0.15	0.29
E1c	100	0.0	0.17	0.20
		0.1	0.14	0.27
		1.0	0.22	0.28
		10.	0.19	nd
		15.	0.17	0.32

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